



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/12, C07K 14/47, A61K 38/17, G01N 33/68, C12Q 1/68, C12N 15/11, 5/10 // (C12N 5/10, C12R 1:91)	A1	(11) International Publication Number: WO 95/18857 (43) International Publication Date: 13 July 1995 (13.07.95)
(21) International Application Number: PCT/US95/00092 (22) International Filing Date: 5 January 1995 (05.01.95) (30) Priority Data: 08/177,571 5 January 1994 (05.01.94) US (71) Applicant: BOARD OF TRUSTEES OF THE UNIVERSITY OF ILLINOIS [US/US]; 352 Henry Administration Build- ing, 506 South Wright Street, Urbana, IL 61801 (US). (72) Inventors: GUDKOV, Andrei; Apartment 2, 5740 South Maryland, Chicago, IL 60637 (US). RONINSON, Igor, B.; 2731 Lincoln Lane, Wilmette, IL 60091 (US). (74) Agent: NOONAN, Kevin, E.; Allegretti & Witcoff, Ltd., Ten South Wacker Drive, Chicago, IL 60606 (US).		(81) Designated States: AU, CA, JP, KR, NO, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: ASSOCIATION OF KINESIN WITH SENSITIVITY TO CHEMOTHERAPEUTIC DRUGS (57) Abstract The invention provides genetic suppressor elements that confer upon a cell resistance to one or more chemotherapeutic drug, methods for identifying and obtaining such elements, and methods of using such elements. The invention also provides cloned genes associated with sensitivity to chemotherapeutic drugs, particularly a cloned human kinesin heavy chain gene involved in resistance to DNA damaging agents.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

ASSOCIATION OF KINESIN WITH SENSITIVITY TO CHEMOTHERAPEUTIC DRUGS

BACKGROUND OF THE INVENTION

5

1. Field Of The Invention

The invention relates to genetic factors associated with sensitivity to chemotherapeutic drugs. More particularly, the invention relates to methods for identifying such factors as well as to uses for such factors. The invention specifically provides genetic suppressor elements derived from mammalian kinesin genes, and therapeutic and diagnostic uses related thereto.

10

2. Summary Of The Related Art

A broad variety of chemotherapeutic agents are used in the treatment of human cancer. For example the textbook *CANCER: Principles & Practice of Oncology*, 2d Edition, (De Vita *et al.*, eds.), J.B. Lippincott Company, Philadelphia, PA (1985) discloses as major antineoplastic agents the plant alkaloids vincristine, vinblastine, and vindesine; the antibiotics actinomycin-D, doxorubicin, daunorubicin, mithramycin, mitomycin C and bleomycin; the antimetabolites methotrexate, 5-fluorouracil, 5-fluorodeoxyuridine, 6-mercaptopurine, 6-thioguanine, cytosine arabinoside, 5-aza-cytidine and hydroxyurea; the alkylating agents cyclophosphamide, melphalan, busulfan, CCNU, MeCCNU, BCNU, streptozotocin, chlorambucil, bis-diaminedichloro-platinum, azetidinylbenzoquinone; and the miscellaneous agents dacarbazine, mAMSA and mitoxantrone.

20

These and other chemotherapeutic agents such as etoposide and amsacrine have proven to be very useful in the treatment of cancer. Unfortunately, some tumor cells become resistant to specific chemotherapeutic agents, in some instances even to multiple chemotherapeutic agents. Such drug resistance or multiple drug resistance can theoretically arise from either the presence of genetic factors that confer resistance to the drugs, or from the absence of genetic factors that confer sensitivity to the drugs. The former type of factors have been identified, and include the multiple drug resistance gene *mdr-1* (see Chen *et al.*, 1986, Cell 47: 381-389). However, the latter type of factor remains largely unknown, perhaps in part because the absence of such factors would tend to be a recessive trait.

30

- 2 -

Identification of genes associated with sensitivity to chemotherapeutic agents is desirable, because the discovery of such genes can lead to both diagnostic and therapeutic approaches for cancer cells and for drug resistant cancer cells, as well as to improvements in gene therapy and rational drug design. Recently, some
5 developments have been made in the difficult area of isolating recessive genetic elements, including those involved in cytotoxic drug sensitivity. Roninson *et al.*, U.S. Patent No. 5,217,889 (issued June 8, 1993) teach a generalized method for obtaining genetic suppressor elements (GSEs), which are dominant negative factors that confer the recessive-type phenotype for the gene to which the particular GSE
10 corresponds. (See also Holzmayer *et al.*, 1992, Nucleic Acids Res. 20: 711-717). Gudkov *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90: 3231-3235 teach isolation of GSEs from topoisomerase II cDNA that induce resistance to topoisomerase II-interactive drugs. Co-pending U.S. Patent Application Serial No. 08/033,986, filed March 3, 1993, discloses the discovery by the present inventors of a novel and
15 unexpected result of experiments performed to identify GSEs isolated from RNA of cells resistant to the anticancer DNA damaging agent, etoposide. This reference discloses that a GSE encoding an antisense RNA homologous to a portion of a mouse kinesin heavy chain gene has the capacity to confer etoposide resistance to cells expressing the GSE. The experiments described in this reference also demonstrate
20 that under-expression of the particular kinesin heavy chain gene disclosed therein was associated with naturally-occurring etoposide resistance in cultures of drug-selected human adenocarcinoma cells. These results were particularly unexpected because the role of kinesin genes in etoposide resistance was unknown in the art prior to the instant inventors' discoveries.

25 The kinesins comprise a family of motor proteins involved in intracellular movement of vesicles or macromolecules along microtubules in eukaryotic cells (see Vale, 1987, Ann. Rev. Cell Biol. 3: 347-378; and Endow, 1991, Trends Biochem. Sci. 16: 221-225 for reviews). Among the family of kinesin genes are encoded kinesin light chains and kinesin heavy chains that assemble to form mature kinesin.
30 A number of kinesin genes have been isolated in the prior art.

Gauger and Goldstein, 1993, J. Biol. Chem. 268: 13657-13666 disclose cloning and sequencing of a *Drosophila* kinesin light chain gene.

- 3 -

Navone *et al.*, 1992, J. Cell. Biol. 117: 1263-1275 disclose cloning and sequencing of a human kinesin heavy chain gene.

Kato, 1991, J. Neurosci. 2: 704-711 disclose cloning and sequencing of a mouse kinesin heavy chain gene.

5 Cyr *et al.*, 1991, Proc. Natl. Acad. Sci. USA 88: 10114-10118 disclose cloning and sequencing of a rat kinesin light chain gene.

McDonald & Goldstein, 1990, Cell 61: 991-1000 disclose isolation of a *Drosophila* kinesin heavy chain gene.

10 Kosik *et al.*, 1990, J. Biol. Chem. 265: 3278-3283 disclose isolation of a squid kinesin heavy chain gene.

The present inventors have demonstrated that a heretofore unexpected gene, a kinesin heavy chain gene, is involved in cellular sensitivity to the anticancer drug etoposide, and that down-regulation of functional expression of this kinesin heavy chain gene is associated with resistance to this drug. Further experiments, disclosed
15 herein, have suggested that the role of kinesin genes in chemotherapeutic drug resistance may not be limited to this single member of the kinesin gene family. These results further underscore the power of the GSE technology developed by these inventors to elucidate unexpected mechanisms of drug resistance in cancer cells, thereby providing the opportunity and the means for overcoming drug
20 resistance in cancer patients. Reagents and methods directed towards such goals are provided in this disclosure.

BRIEF SUMMARY OF THE INVENTION

The invention provides genetic suppressor elements (GSEs) that are random
25 fragments derived from genes associated with sensitivity to chemotherapeutic drugs, and that confer resistance to chemotherapeutic drugs and DNA damaging agents upon cells expressing such GSEs. The invention specifically provides GSEs derived from cDNA and genomic DNA encoding kinesin genes. Diagnostic assays useful in determining appropriate candidate cancer patients bearing tumors likely to be
30 successfully reduced or eliminated by administration of particular anticancer treatment modalities, including chemotherapeutic drugs and other DNA damaging agents, are provided by the invention, on the basis of levels of kinesin gene

- 4 -

expression in the tumor cells borne by such cancer patients. *In vitro* drug screening and rational drug design methods are also within the scope of the instant disclosure.

The invention is based in part on the discoveries disclosed in co-pending U.S. Patent Application Serial No. 08/033,086, filed March 3, 1993 and incorporated by reference, providing a method for identifying and isolating GSEs that confer resistance to any chemotherapeutic drug for which resistance is possible. Particularly provided herein are methods for identifying GSEs derived from any kinesin gene, said GSEs being capable of conferring resistance to DNA damaging agents on cells expressing the GSEs. This method utilizes chemotherapeutic drug selection of cells that harbor clones from a random fragment expression library derived from kinesin-specific cDNA, and subsequent rescue of library inserts from drug-resistant cells. In a second aspect, the invention provides GSEs comprising oligonucleotides and/or peptides derived from kinesin genes that function as GSEs *in vivo* and confer on cells expressing said GSEs resistance to DNA damaging agents, including certain chemotherapeutic drugs. In a third aspect, the invention provides a method for obtaining GSEs having optimized suppressor activity for a kinesin gene associated with sensitivity to a chemotherapeutic drug. This method utilizes chemotherapeutic drug selection of cells that harbor clones from a random fragment expression library derived from DNA of a kinesin gene associated with sensitivity to that chemotherapeutic drug, and subsequent rescue of the library inserts from drug resistant cells. Particularly and preferably provided are such optimized GSEs derived from a mouse or human kinesin gene. In a fourth aspect, the invention provides synthetic peptides and oligonucleotides that confer upon cells resistance to DNA damaging agents, including certain chemotherapeutic drugs. These synthetic peptides and oligonucleotides are designed based upon the sequence of a drug-resistance conferring GSE derived from a mouse or human kinesin gene according to the invention.

In a fifth aspect, the invention provides a diagnostic assay for tumor cells that are resistant to one or more therapeutic DNA damaging agents and, at the same time, sensitive to therapeutic anti-microtubular agents, due to the absence of expression or under-expression of a kinesin gene. This diagnostic assay comprises quantitating the level of expression of any particular kinesin gene product in a particular tumor cell

- 5 -

sample to be tested, and comparing the expression levels so obtained with a standardized set of cell lines expressing varying amounts of kinesin gene mRNA and/or protein and having different degrees of resistance to chemotherapeutic drugs and DNA damaging agents associated with their levels of kinesin gene expression.

5 In preferred embodiments, such a standardized set of cell lines is matched by tissue type with the tissue type of the tumor cells to be evaluated.

In a sixth aspect, the invention provides methods for determining the appropriateness of candidates for particular cancer chemotherapeutic treatment modalities. In one preferred embodiment, the invention provides a means for
10 determining whether a cancer patient is an appropriate candidate for treatment with DNA damaging chemotherapeutic drugs or other DNA damaging agents such as radiation, the method determining whether a kinesin gene, such as the kinesin heavy chain gene disclosed herein and in co-pending U.S. Patent Application Serial No. 08/033,086, is over-expressed or under-expressed in tumor cells borne by a cancer
15 patient, relative to a standardized set of cell lines as disclosed herein. Using this method, appropriate candidates for treatment with DNA damaging agents, including certain chemotherapeutic drugs, will be those patients whose tumor cells over-express the kinesin gene. In another embodiment, the invention provides a means for determining whether a cancer patient is an appropriate candidate for treatment with
20 anti-microtubular chemotherapeutic drugs. Using this aspect of the method, appropriate candidates for anti-microtubular agent treatment will be those patients whose tumor cells under-express the kinesin gene compared with expression levels in a standardized set of cell lines. In a particularly useful embodiment of this aspect of the invention, potential candidate cancer patients for treatment with anti-
25 microtubular anticancer agents will have failed or proven resistant to a course of cancer chemotherapy using DNA damaging agents.

In a seventh aspect, the invention provides a starting point for the rational design of pharmaceutical products that are useful against tumor cells that are resistant to chemotherapeutic drugs. By examining the structure, function, localization and
30 pattern of expression of kinesin genes associated with resistance to DNA damaging agents and sensitivity to anti-microtubular chemotherapeutic drugs, strategies can be developed for creating pharmaceutical products that will overcome drug resistance

- 6 -

in tumor cells in which such kinesin genes are either over-expressed or under-expressed.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show a scheme for construction of a random fragment expression library (RFEL) from NIH 3T3 cDNA. Figure 1A shows the overall construction scheme. Figure 1B shows normalization of the cDNA fragments. In Figure 1B, t represents total unfractionated cDNA, s and d represent the single-stranded and double-stranded fractions separated by hydroxyapatite, time points indicate the period of reannealing, and tubulin, *c-myc*, and *c-fos* indicate the probes used in Southern hybridization with the total, single-stranded and double-stranded fractions.

Figure 2 shows the structure of the LNCX vector and the adaptor used in cDNA cloning. The nucleotide sequences are shown for the ATG-sense (SEQ.ID.No.:1) and ATG-antisense (SEQ.ID.No.:2) strands of the adaptor.

Figure 3 shows the overall scheme for selecting cell lines containing chemotherapeutic drug resistance-conferring GSEs and rescuing the GSEs from these cells.

Figures 4A and 4B show etoposide resistance conferred by preselected virus (Figure 4A) and PCR analysis of the selected and unselected populations (Figure 4B). Figure 4C illustrates a schema for recloning individual PCR-amplified fragments into the LNCX vector in the same position and orientation as in the original plasmid.

Figures 5A and 5B show resistance to various concentrations of etoposide, conferred upon the cells by the GSE anti-*khcs* under an IPTG-inducible promoter (Figure 5A), and the scheme for this experiment (Figure 5B).

Figure 6 shows the nucleotide sequence of the GSE anti-*khcs* (SEQ.ID.No.:3).

- 7 -

Figure 7 shows the nucleotide sequence of most of the coding region of the mouse *khcs* cDNA (SEQ.ID.No.:4).

Figures 8A through 8D show the dot matrix alignments of *khcs* protein sequence deduced from the nucleotide sequence in Figure 7 with kinesin heavy chain sequences from human (Figure 8A), mouse (Figure 8B), fruit fly (Figure 8C), and GSE-C (Figure 8D).

Figures 9A and 9B illustrates the experimental protocol for drug-selected production of kinesin-derived GSEs (Figure 9A) and the structure of the adaptors used for the preparation of a random fragment *KHCS* cDNA library (Figure 9B).

Figure 10 shows etoposide resistance in HT1080 cells carrying insert-free vector virus or a random fragment library of human *KHCS* cDNA.

Figure 11 shows the effects of different drugs on 4-day growth of NIH 3T3 cells infected with insert-free vector virus or with a virus encoding anti-*khcs*. Cell growth in the absence of the drug differed less than 5% for the compared populations. Drug concentrations are given in ng/mL. A representative series of parallel assays, carried out in triplicate, is shown.

Figure 12 shows growth of cells carrying anti-*khcs* (solid lines) and control cells (broken lines) after treatment with colchicine or vinblastine. Cells were incubated with the drugs for 4 days, followed by 2 or 4 days in drug-free media, as indicated. Cell growth, presented in arbitrary units, was evaluated by methylene blue staining.

Figure 13 shows a kinetic analysis of cell growth of anti-*khcs* GSE-carrying cells (black lines) and control cells (grey lines) incubated with different drugs. Cell growth was measured as described for Figure 13. Cells were plated and one day later (indicated by the first arrow) the indicated drugs were added at concentrations as described. Four days later (indicated by the second arrow), the drugs were removed from some of the plates. Solid lines indicate cell growth in the continuous presence of the drug, and broken lines indicate cell growth after removal of the drug.

Figure 14 demonstrates increased immortalization of primary mouse embryo fibroblasts by infection with the LNCX vector containing the anti-*khcs* GSE, relative to cells infected with the LNCX vector alone or uninfected (control) cells.

- 8 -

Figure 15 demonstrates increased immortalization of primary human skin fibroblasts by infection with the LNCX vector containing the anti-*khcs* GSE (anti-*khcs*) at the 4th passage after infection, relative to human skin fibroblasts in growth crisis (control).

5 Figure 16 shows cDNA-PCR quantitative analysis of expression of the human *khcs* gene in various unselected and etoposide-selected human HeLa cells. Lanes a shows results for clone CS(O), lands a' for clone CX(200), lanes b for clone Σ /11(O), lanes b' for clone Σ 11 (1000), lanes c for clone 6(O), lanes c' for clone 6(1000), lanes d for clone Σ 20(O) and lanes d' for clone Σ 20 (1000). The numbers
10 in parentheses for each clone name indicate the concentration of etoposide (ng/ml) present in the growth media. Bands indicative of *khcs* expression are shown along with bands for β -2 macroglobulin expression as an internal control.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

15 The invention relates to means for suppressing specific gene functions that are associated with sensitivity and resistance to chemotherapeutic drugs. The invention provides genetic suppressor elements (GSEs) derived from kinesin genes that have such suppressive effect and thus confer resistance to DNA damaging agents including chemotherapeutic drugs. The invention further provides methods for identifying such
20 GSEs, as well as methods for their use.

For the purposes of this invention, the term "kinesin gene" will be understood to encompass any kinesin gene, particularly mammalian, and preferably mouse or human, kinesin genes. Kinesins comprise a family of related genes encoding a number of related motor proteins involved in intracellular movement of vesicles or
25 macromolecules along microtubules in eukaryotic cells. (see Background of the Invention). The mature, functional kinesin molecule is comprised of products of a kinesin heavy chain gene and a kinesin light chain gene. The instant invention encompasses GSEs derived from both kinesin light chain and kinesin heavy chain genes. The invention specifically is intended to contain within its scope all kinesin
30 genes and GSEs derived therefrom that are capable of causing resistance or sensitivity to DNA damaging agents.

- 9 -

The DNA damaging agents that fall within the scope of this invention are all DNA damaging agents, including but not limited to ionizing and ultraviolet radiation, and certain chemotherapeutic drugs, including amsacrine, etoposide, doxorubicin (Adriamycin), cisplatin, and camptothecin.

5 In a first aspect, the invention provides a GSE derived from the cDNA of a mouse kinesin heavy chain gene isolated from a normalized, random fragment expression library made from total cellular mRNA from NIH 3T3 cells and isolated on the basis of its ability to confer resistance to the topoisomerase II drug, etoposide (described in Examples 1-4 herein and co-pending U.S. Patent Application Serial No.
10 08/033,086, filed March 3, 1993 and incorporated by reference). Prior to the discovery by the present inventors, there was no suspicion that kinesin was in any way implicated in etoposide sensitivity. These results demonstrated the ability of the general method for identifying GSEs to provide much new and surprising information about the genetic basis for resistance to chemotherapeutic drugs.

15 In addition, the kinesin-derived GSE conferring resistance to etoposide caused cellular effects suggesting that kinesin may be involved in programmed cell death. The method according to this aspect of the invention therefore also provides valuable information about the genetic basis for senescence and cell death. This may have important implications for studying genes involved in development, since GSEs used
20 to identify genes associated with chemotherapeutic drug resistance or senescence can also be expressed as transgenes in embryos to determine the role of such genes in development. The elucidation of the structure of the mouse kinesin heavy chain gene corresponding to this drug resistance-related GSE is described in Example 5 and functional analyses of the drug resistance capacity of this GSE is disclosed in
25 Example 7.

In a second aspect, the invention provides a method for identifying kinesin gene-derived GSEs that confer resistance to a DNA damaging agent. The GSEs identified by this method will be homologous to a kinesin gene. For purposes of the invention, the term "homologous to" a kinesin gene has two different meanings,
30 depending on whether the GSE acts through an antisense mechanism or antigene mechanism (*i.e.*, through a mechanism of interference at the protein level). In the former case, a GSE that is an antisense or antigene oligonucleotide or polynucleotide

is homologous to a gene if it has a nucleotide sequence that hybridizes under physiological conditions to the gene or its mRNA transcript by Hoogsteen or Watson-Crick base-pairing. In the latter case, a GSE that interferes with a protein molecule is homologous to the gene encoding that protein molecule if it has an amino acid sequence that is the same as that encoded by a portion of the gene encoding the protein, or that would be the same, but for conservative amino acid substitutions. In either case, as a practical matter, whether the GSE is homologous to a gene is determined by assessing whether the GSE is capable of inhibiting or reducing the function of the gene; in particular, any kinesin gene, preferably any mouse or human kinesin gene, as disclosed herein.

The method according to this aspect of the invention comprises the step of screening a kinesin-specific cDNA or kinesin-specific genomic DNA random fragment expression library phenotypically to identify clones that confer resistance to a DNA damaging agent such as certain chemotherapeutic drugs. Preferably, the library of random fragments of kinesin-specific cDNA or kinesin-specific genomic DNA is cloned into a retroviral expression vector. In this preferred embodiment, retrovirus particles containing the library are used to infect cells and the infected cells are tested for their ability to survive in a concentration of a DNA damaging agent that kills uninfected cells. Preferably, the inserts in the library will range from about 100 b.p. to about 700 b.p. and more preferably, from about 200 b.p. to about 500 b.p. Once a clonal population of cells that are resistant to the DNA damaging agent has been isolated, the library clone encoding the GSE is rescued from the cells. At this stage, the nucleotide sequence of the insert of the expression library may be determined; in clones derived from a kinesin gene-specific cDNA random fragment expression library, the nucleotide sequence is expected to be homologous to a portion of the kinesin gene cDNA nucleotide sequence. Alternatively, the rescued library clone may be further tested for its ability to confer resistance to DNA damaging agents and chemotherapeutic drugs in additional transfection or infection and selection assays, prior to nucleotide sequence determination. Determination of the nucleotide sequence, of course, results in the identification of the GSE. This method is further illustrated in Example 6.

- 11 -

Thus, the invention provides a method for obtaining kinesin gene-derived GSEs having optimized suppressor activity. By screening a random fragment expression library made exclusively from kinesin gene-specific fragments, a much greater variety of GSEs derived specifically from the kinesin gene can be obtained, compared with a random fragment library prepared from total cDNA as in Example 1. Consequently, the likelihood of obtaining optimized GSEs, *i.e.*, those kinesin-derived GSEs conferring an optimal level of resistance to a chemotherapeutic drug, is maximized using the single gene random fragment library approach, as is shown in greater detail in Example 6.

10 An additional feature of this aspect of the invention is the production of a multiplicity of kinesin-specific GSEs by drug selection of cells producing infectious retroviral embodiments of the kinesin-derived GSEs of the invention. In this aspect, ecotropic cells infected with a kinesin cDNA or kinesin genomic DNA-specific random fragment expression library are subjected to selection with a DNA damaging agent, preferably and most practically a chemotherapeutic drug such as etoposide. A population of resistant clones are thereby obtained, each containing a drug resistance-conferring, kinesin-derived GSE. Since these cells are capable of producing infection retroviral embodiments of the GSEs of the invention, a multiplicity of kinesin-derived GSEs, pre-selected for the ability to confer drug resistance, can be easily and efficiently produced.

20 In a fourth aspect, the invention provides synthetic peptides and oligonucleotides that are capable of inhibiting the function of kinesin genes associated with sensitivity to chemotherapeutic drugs. Synthetic peptides according to the invention have amino acid sequences that correspond to amino acid sequences encoded by GSEs according to the invention. Synthetic oligonucleotides according to the invention have nucleotide sequences corresponding to the nucleotide sequences of GSEs according to the invention. Once a GSE has been discovered and sequenced, and its orientation is determined, it is straightforward to prepare an oligonucleotide corresponding to the nucleotide sequence of the GSE (for antisense-oriented GSEs) or amino acid sequence encoded by the GSE (for sense-oriented GSEs). In certain embodiments, such synthetic peptides or oligonucleotides may have the complete sequence encoded by the GSE or may have only part of the

- 12 -

sequence present in the GSE, respectively. In certain other embodiments, the peptide or oligonucleotide may have only a portion of the GSE-encoded or GSE sequence. In such latter embodiments, undue experimentation is avoided by the observation that many independent GSE clones corresponding to a particular gene will have the same 5' or 3' terminus, but generally not both. This suggests that many GSE's have one critical endpoint, from which a simple walking experiment will determine the minimum size of peptide or oligonucleotide necessary to inhibit gene function. For peptides, functional domains as small as 6-8 amino acids have been identified for immunoglobulin binding regions. Thus, peptides or peptide mimetics having these or larger dimensions can be prepared as GSEs. For antisense oligonucleotides, inhibition of gene function can be mediated by oligonucleotides having sufficient length to hybridize to their corresponding mRNA under physiological conditions. Generally, oligonucleotides having about 12 or more bases will fit this description. Preferably, such oligonucleotides will have from about 12 to about 100 nucleotides. As used herein, the term oligonucleotide includes modified oligonucleotides having nuclease-resistant internucleotide linkages, such as phosphorothioate, methylphosphonate, phosphorodithioate, phosphoramidate, phosphotriester, sulfone, siloxane, carbonate, carboxymethylester, acetamidate, carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate and bridged phosphorothioate internucleotide linkages. The synthesis of oligonucleotides containing these modified linkages is well known in the art. (See, e.g., Uhlmann and Peyman, 1990; Chemical Reviews 90: 543-584; Schneider and Banner, 1990, Tetrahedron Letters 31: 335). The term oligonucleotides also includes oligonucleotides having modified bases or modified ribose or deoxyribose sugars.

In a fifth aspect, the invention provides dominant selectable markers that are useful in gene co-transfer studies. Since GSEs according to the invention confer resistance to chemotherapeutic drugs, the presence of a vector that expresses the GSE can readily be selected by growth of a vector-transfected cell in a concentration of the appropriate cytotoxic drug that would be cytotoxic in the absence of the GSE. GSEs according to the invention are particularly well suited as dominant selectable markers because their small size allows them to be easily incorporated along with a gene to be co-transferred even into viral vectors having limited packaging capacity.

- 13 -

In a sixth aspect, the invention provides a diagnostic assay for tumor cells that are resistant to one or more DNA damaging agents, including certain chemotherapeutic drugs, due to the absence of expression or the under-expression of a kinesin gene. In particular, the class of DNA damaging agents resistance to which involves under-expression of a kinesin gene includes but is not limited to cisplatin, etoposide and camptothecin. To determine whether absence of expression or under-expression of a kinesin gene is a naturally occurring, and thus medically significant basis for chemotherapeutic drug resistance, human tumor cells can be treated with cytotoxic quantities of an appropriate chemotherapeutic drug to select for spontaneous drug resistant mutants. These mutants can then be assessed for their level of expressing of the particular gene of interest. Absence of expression or significantly reduced expression indicates a natural mechanism of chemotherapeutic drug resistance. The description of such an experiment, disclosing that under-expression of the human kinesin heavy chain gene disclosed herein is associated with naturally-occurring resistance to the chemotherapeutic drug etoposide in cultures of etoposide-resistant human adenocarcinoma (HeLa) cells, is disclosed in Example 11 herein and in co-pending U.S. Patent Application Serial No. 08/033,086, filed March 3, 1993 and incorporated by reference. In a preferred embodiment of this assay, a standardized set of tissue-specific cell lines, wherein the levels of drug resistance and kinesin gene expression have been quantitated and correlated with each other, are provided for tumors from each tissue type to be assayed.

Alternatively, and preferably, collections of naturally occurring treatment-responding and non-responding tumor tissue samples can be examined for expression levels of kinesin genes, and correlations established between treatment outcome, and presumably the drug-resistant mechanisms thereof, and kinesin gene expression.

Accordingly, such reduced or absent expression can be the basis for a diagnostic assay for tumor cell resistance to a DNA damaging agent or chemotherapeutic drug or drugs of interest. A first embodiment of a diagnostic assay according to this aspect of the invention utilizes an oligonucleotide or oligonucleotides that is/are homologous to the sequence of a kinesin gene. In this embodiment, RNA is extracted from a tumor sample, and RNA specific for a particular kinesin gene is quantitated by standard filter hybridization procedures, an

- 14 -

RNase protection assay, or by quantitative cDNA-PCR (see Noonan *et al.*, 1990, Proc. Natl. Acad. Sci. USA 87: 7160-7164). In a second embodiment of a diagnostic assay according to this aspect of the invention, antibodies are raised against a synthetic peptide having an amino acid sequence that is identical to a portion of the kinesin heavy chain or kinesin light chain protein. Antibodies specific for the human kinesin heavy chain have in fact been disclosed (see Navone *et al.*, *supra*). These antibodies are then used in a conventional quantitative immunoassay (e.g., RIA or immunohistochemical assays) to determine the amount of the gene product of interest present in a sample of proteins extracted from the tumor cells to be tested, or on the surface or at locations within the tumor cells to be tested.

A particular utility for such diagnostic assays of this invention are their clinical use in making treatment decisions for the alleviation of malignant disease in humans. For example, a determination that the kinesin heavy chain gene of this invention is under-expressed in a tumor compared with the levels of expression found in normal cells comprising that tissue would suggest that a patient nearing such a tumor might be a poor candidate for therapeutic intervention using DNA damaging agents, since it would be expected that such kinesin under-expressing cells of the tumor would be resistant to such agents. Similarly, tumor cells which fortuitously over-express the kinesin heavy chain gene of the invention would be expected to be sensitive to such agents and thus to be susceptible to tumor cell killing by these agents. On the other hand, the instant disclosure provides experimental evidence that kinesin heavy chain gene under-expressors are sensitive to the cytotoxic action of anti-microtubular agents, including for example colchicine, colcemide, vinblastine, vincristine and vindesine. These results suggest that patients bearing tumors whose cells under-express the kinesin heavy chain gene of the present invention may be responsive to treatment with DNA damaging agents. The present invention thus enables intelligent and informed therapeutic intervention based on properties of an individual cancer patient's tumor resistance or sensitivity to DNA damaging agents and other chemotherapeutic treatment modalities, where treatment choices can be made prior to initiation of treatment based on mechanisms of resistance specific for DNA damaging agents and mediated by kinesin heavy chain gene over- or under-expression. Particularly useful in this aspect of the invention are kinesin-specific

- 15 -

antibodies, such as the anti-kinesin heavy chain antibodies described in Navone, *et al.*, *supra*, for detection of kinesin expression levels in tumor samples.

In a seventh aspect, the invention provides a starting point for the rational design of pharmaceutical products that can counteract resistance by tumor cells to
5 chemotherapeutic drugs.

Understanding the biochemical function of the kinesin genes that are involved in drug sensitivity is likely to suggest pharmaceutical means to stimulate or mimic the function of such genes and thus augment the cytotoxic response to anticancer drugs. One may also be able to up-modulate gene expression at the level of
10 transcription. This can be done by cloning the promoter region of each of the corresponding kinesin genes and analyzing the promoter sequences for the presence of *cis* elements known to provide the response to specific biological stimulators. Due to the structure of the kinesins in eukaryotic cells, *i.e.*, comprised of both kinesin heavy chain and kinesin light chain proteins, coordinate up-regulation of the
15 expression of both of the appropriate kinesin light chain and heavy chain proteins would be required for efficacious therapeutic intervention based on modulating expression of kinesin genes.

Alternatively, kinesin expression in a cancer cell can be increased by co-introduction of recombinant expression constructs encoding functional, full-length
20 copies of a kinesin heavy chain and a kinesin light chain, whereby coordinate co-expression of such exogenous kinesins would result in increased expression of functional kinesin molecules in the cancer cells.

The protein structure deduced from the cDNA sequence can also be used for computer-assisted drug design, to develop new drugs that affect this protein in the
25 same manner as the known anticancer drugs. The purified protein, produced in a convenient expression system, can also be used as the critical component of *in vitro* biochemical screen systems for new compounds with anticancer activity. Accordingly, mammalian cells that express chemotherapeutic drug resistance-conferring GSEs according to the invention are useful for screening compounds for
30 the ability to overcome drug resistance. As with pharmaceutical intervention methods, both kinesin light chains and heavy chains should be present in such *in*

- 16 -

vitro screening systems in amounts capable of reconstituting mature kinesin molecules *in vitro*.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and
5 the claims.

EXAMPLE 1

Generation Of a Normalized Random Fragment cDNA Library In A Retroviral Vector

10 A normalized cDNA population was prepared using a modification of the protocol of Patanjali *et al.* (1991, Proc. Natl. Acad. Sci. USA 88: 1943-1947), illustrated in Figure 1A and 1B. Poly(A)⁺ RNA was extracted from NIH 3T3 cells. To obtain mRNAs for different genes expressed at various stages of the cell growth, one half of the RNA was isolated from a rapidly growing culture and the other half
15 from quiescent cells that had reached complete monolayer confluence. To avoid overrepresentation of the 5'-end sequences in a randomly primed cDNA population, RNA was fragmented by boiling to an average size range of 600-1,000 nucleotides. These RNA fragments were then used for preparing randomly primed double-stranded cDNA. This randomly primed cDNA was then ligated to a synthetic
20 adaptor providing ATG codons in all three possible reading frames and in a proper context for translation initiation. The structure of the adaptor (see Figure 2) determined its ligation to the blunt-ended fragments of the cDNA in such a way that each fragment started from initiation codons independently from its orientation. The adaptor was not supplied with termination codons in the opposite strand since the
25 cloning vector pLNCX, contained such codons immediately downstream of the cloning site. (This vector has been described by Miller and Rosman, 1989, Biotechniques 7: 980-986.) The ligated mixture was amplified by PCR, using the "sense" strand of the adaptor as a PCR primer, (in contrast to the method of Patanjali *et al.*, which utilized cloning the initial cDNA preparation into a phage
30 vector and then using vector-derived sequences as PCR primers to amplify the cDNA population.) The PCRs were carried out in 12 separate reactions that were subsequently combined, to minimize random over- or under-amplification of specific sequences and to increase the yield of the product. The PCR-amplified mixtures was

- 17 -

size-fractionated by gel electrophoresis, and 200-500 bp fragments were selected for subsequent manipulations, (in contrast to Patanjali's fragment size range of from 400 to 1,600 bp.)

For normalization, the cDNA preparation was denatured and reannealed, using different time points for reannealing, as described by Patanjali *et al.*, *supra*, and shown in Figure 1A. The single-stranded and double-stranded DNAs from each reannealed mixture were separated by hydroxyapatite chromatography. The single-stranded DNA fractions from each time point of reannealing were PCR-amplified using the adaptor-derived primer and analyzed by Southern hybridization for the relative abundance of different mRNA sequences. The fraction that contained similar proportions of tubulin, *c-myc* and *c-fos* cDNA sequences (see Figures 1A and 1B), corresponding to high-, medium- and low-expressed genes, respectively, was used for the library preparation.

The normalized cDNA preparation was cloned into a *Clal* site of the MoMLV-based retroviral vector pLNCX, which carries the *neo* (G418 resistance) gene, transcribed from the promoter contained in the retroviral long terminal repeat (LTR), and which expresses the inserted sequence from a strong promoter of the cytomegalovirus (CMV) (see Figure 2). The ligation mixture, divided into five portions, was used for five subsequent large-scale transformations of *E. coli*. The transformed bacteria were plated on the total of 500 agar plates (150 mm in diameter) and the plasmid population (18 mg total) was isolated from the colonies washed off the agar. A total of approximately 5×10^7 clones were obtained, more than 60% of which carried the inserts of normalized cDNA, as estimated by PCR amplification of inserts from 50 randomly picked colonies. These results demonstrate the feasibility of generating a normalized cDNA library of as many as 3×10^7 recombinant clones in a retroviral plasmid expression vector.

EXAMPLE 2

Transduction Of A Retroviral Random Fragment Library Into Virus-Packaging Cell Lines And NIH 3T3 Cells

The plasmid library prepared according to Example 1 was converted into a mixture of retroviral particles by transfection into virus-packaging cells (derivatives of NIH 3T3) that express retroviral virion proteins. (Examples of such cell lines have

- 18 -

been described by Markowitz *et al.*, 1988, Virology 167: 400-406.) Ecotropic and amphotropic virus-packaging cell lines, GP+ E86 and GP+envAm12, respectively, were mixed at a 1:1 ratio and 10^7 cells of this mixture were transfected with the plasmid library under standard calcium phosphate coprecipitation conditions. This transfection resulted in the packaging and secretion of ecotropic and amphotropic virus particles, which rapidly spread through the packaging cell population, since ecotropic viruses are capable of infecting amphotropic packaging cells and vice versa. The yield of the virus, as measured by the number of G418-resistant colonies obtained after the infection of NIH 3T3 cells, reached 10^5 infectious units per 1 mL of media during the stage of transient transfection (1-3 days), then decreased (4-8 days) and then rapidly increased due to the expression of proviral genomes that became stably integrated in most of the packaging cells. The yield of the virus 9-12 days after transfection reached $>10^6$ per 1 mL of media supernatant. At this stage, the library showed fairly even representation of different fragments, but at later stages individual virus-producing clones began to predominate in the population, leading to uneven representation of cDNA-derived inserts. The uniformity of sequence representation in the retroviral population was monitored by rapid extraction of DNA from cells infected with the virus-containing supernatant, followed by PCR amplification of inserts. The inserts were analyzed first by the production of a continuous smear in ethidium bromide-stained agarose gel and then by Southern hybridization with different probes, including topoisomerase II, *c-myc* and tubulin. As long as each gene was represented by a smear of multiple fragments, the representativity of the library was considered to be satisfactory.

In other experiments, for transducing the random-fragment normalized cDNA library into NIH 3T3 cells, without loss of representativity, NIH 3T3 cells were infected either with a virus produced at the transient stage of transfection (days 1-3), or with the high-titer virus collected 10-12 days after transfection. In the latter case, 100 ml of viral suspension contained more than 10^8 infectious units. In the case of the "transient" virus, NIH 3T3 cells were infected with at least 10^7 recombinant retroviruses by using 500 ml of media from virus-producing cells (five rounds of infection, 100 ml of media in each). These results demonstrate the feasibility of

- 19 -

converting a large and complex random fragment library into retroviral form and delivering it to a non-packaging cell line without loss of complexity.

EXAMPLE 3

Isolation of GSEs Conferring Resistance To The Chemotherapeutic Drug Etoposide

5

The overall scheme for the selection of GSEs conferring etoposide resistance is illustrated in Figure 3. This selection was carried out directly on virus-producing packaging cells, in the expectation that cells whose resistant phenotype is caused by the GSE expression will produce virus particles carrying such a GSE. The mixture of amphotropic and ecotropic packaging cells was transfected with the cDNA library in the LNCX vector, prepared according to Example 1, and the virus was allowed to spread through the population for 9 days. Analysis of a small part of the population for G418 resistance showed that practically 100% of the cells carried the *neo*-containing provirus. The cells were then exposed to 350ng/mL etoposide for 15 days and then allowed to grow without drug for two more weeks. No difference was observed between the numbers of colonies obtained in the experiment and in the control (uninfected cells or cells infected with the insert-free LNCX virus) after etoposide selection. The virus present in the media supernatant of the surviving cells was then used to infect NIH 3T3 cells followed by etoposide selection using essentially the same protocol. NIH 3T3 cells infected with the library-derived virus produced by packaging cells that were selected with etoposide showed a major increase in the number of etoposide-resistant cells relative to the control cells infected with the insert-free LNCX virus, indicating the presence of biologically active GSEs in the preselected virus population (see Figure 4A).

20

25

The proviral inserts contained in the etoposide-selected NIH 3T3 cells were analyzed by PCR. This analysis (see Figure 4B) showed an enrichment for specific fragments, relative to the unselected population of the infected cells. Individual PCR-amplified fragments were recloned into the LNCX vector in the same position and orientation as in the original plasmid, as illustrated in Figure 4C. A total of 42 proviral inserts, enriched after etoposide selection, were thus recloned, and tested either in batches or individually for the ability to confer increased etoposide resistance after retroviral transduction into NIH 3T3 cells. Three non-identical

30

- 20 -

clones were found to induce etoposide resistance, indicating that they contained biologically active GSEs. These GSEs were named anti-*khcs*, VPA and VP9-11. Etoposide resistance induced by the clone named anti-*khcs* is illustrated in Figure 5A.

The ability of one of the anti-*khcs* GSE to induce etoposide resistance was further documented by using the isopropyl β -D-thiogalactopyranoside (IPTG)-inducible promoter/activator system, as described by Baim *et al.* (1991, Proc. Natl. Acad. Sci. USA 88: 5072-5076). The components of this system include an enhancer-dependent promoter, combined in *cis* with multiple repeats of the bacterial *lac* operator, and a gene expressing LAP265, an artificial regulatory protein derived from the *lac* repressor and a mammalian transcriptional activator. The anti-*khcs* GSE was cloned into the plasmid pX6.CLN, which contains the inducible promoter used by Baim *et al.*, *supra* (a gift of Dr. T. Shenk) which expresses the inserts from an enhancerless SV40 early gene promoter supplemented with 21 repeats of the *lac* operator sequence. The resulting plasmid, which contains no selectable markers, was co-transfected into NIH 3T3 cells together with the LNCX plasmid carrying the *neo* gene. The mass population of G418-selected transfectants, along with control cells transfected with the insert-free vector, was exposed to increasing concentrations of etoposide, in the presence or in the absence of 5 mM IPTG. Even though the co-transfection protocol usually leads to the integration of the GSE in only a fraction of the G418-resistant cells, transfection with anti-*khcs* resulted in a clearly increased etoposide resistance, which was dependent on IPTG (see Figure 5B).

EXAMPLE 4

Sequence Analysis of GSEs Conferring Resistance To The Chemotherapeutic Drug Etoposide

The GSE anti-*khcs*, cloned as described in Example 3, was sequenced by the standard dideoxy sequencing procedure, and the deduced sequence is shown in Figure 6. The nucleotide sequence of the "sense" and "antisense" strands, as well as amino acid sequence of the predicted peptides encoded by each of these strands, were analyzed for homology to the nucleic acid and protein sequences present in the National Center for Biotechnology Information data base, using the BLAST network program for homology search. The sequence corresponding to the "antisense" strand

- 21 -

of the anti-*khcs* GSE, showed strong homology with several genes encoding the heavy chain of kinesins, a family of microtubule motor proteins involved in intracellular movement of organelles or macromolecules along the microtubules of eukaryotic cells. The highest homology was found with the human kinesin heavy chain (KHC) gene, as described by Navone *et al.* (1992, J. Cell Biol. 117: 1263-1275). Anti-*khcs* therefore encodes antisense RNA for a mouse *khc* gene, which we have termed *khcs* for *khc* associated with sensitivity (to drugs) or senescence. We refer to the kinesin molecule, formed by the associate of the KHCS protein with kinesin light chains, as kinesin-S, to distinguish it from the other kinesins present in mammalian cells. These results demonstrate that chemotherapeutic drug selection for GSEs can lead to the discovery of novel genetic elements, and can also reveal roles of genes in drug sensitivity that had never before been suspected.

EXAMPLE 5

Cloning And Analysis Of The Gene From Which Anti-*khcs* GSE Gene Was Derived

The anti-*khcs* GSE isolated in Example 3 was used as a probe to screen 400,000 clones from each of two cDNA libraries in the lambda gt10 vector. These libraries were prepared by conventional procedures from the RNA of mouse BALB/c 3T3 cells, either unsynchronized or at G₀ → G₁ transition, as described by Lau and Nathans (1985, EMBO J. 4: 3145-3151 and 1987, Proc. Natl. Acad. Sci. USA 84: 1182-1186, a gift of Dr. L. Lau). Screening of the first library yielded no hybridizing clones, but two different clones from the second library were found to contain anti-*khcs* sequences. These clones were purified and sequenced. Sequence analysis showed that we have isolated the bulk of the mouse *khcs* cDNA, corresponding to 796 codons (the full-length human KHC cDNA encodes 963 amino acids). This sequence is shown in Figure 7; an additional 252 nucleotides encoding 84 amino acids from the amino terminus have been determined from 5'-specific cDNA isolated using the "anchored PCR" technique, as described by Ohara *et al.* (1989, Proc. Natl. Acad. Sci. USA 86: 5763-5677.) Additional missing 3' terminal sequences are currently being isolated using this technique.

- 22 -

The dot-matrix alignment of the sequenced portion of the *khcs* protein with previously cloned KHC proteins from the human (*see Navone et al.*, 1992, J. Cell. Biol. 117: 1263-1275), mouse (*see Kato*, 1991, J. Neurosci. 2: 704-711), and *Drosophila* (McDonald & Goldstein, 1990, Cell 61: 991-1000) is shown in Figures 5 8A through 8C; homology to GSE-C is shown as a control (Figure 8D). The portion corresponding to the anti-*khcs* GSE, is shown in brackets. The *khcs* gene is most highly homologous to the human gene (97% amino acid identity), suggesting that the human *KHC* (*KHCS*) gene is functionally equivalent to the mouse *khcs*. The alignment also shows that the anti-*khcs* GSE corresponds to the region which is the 10 most highly diverged between different kinesins (shown in the Figure by brackets around these sequences.)

EXAMPLE 6

15 Generation of a Random Fragment *KHCS* cDNA Retroviral Library and Isolation of *KHCS*-derived GSEs

As described in Example 5 above, the murine *khcs* gene is highly homologous to the human *KHC* (or *KHCS*) gene described by Navone *et al.* (1992, J. Cell Biol. 117: 1263-1275). The functional equivalence of these genes was also suggested by the observation that the levels of *KHCS* mRNA are decreased in human cells selected 20 for etoposide resistance (*see Example 8*). To determine conclusively that the human *KHCS* gene represents the functional equivalent of the mouse *khcs*, it was determined whether any random fragment of human *KHCS* cDNA could function as an etoposide-resistance GSE.

A library of random DNAaseI-generated fragments of a full-length human 25 *KHCS* cDNA (2.9 kb in length; provided by Dr. R. Vale, University of California at San Francisco) was generated essentially as described above for topoisomerase II cDNA (*see Example 1* in co-pending U.S. Patent Application Serial No. 08/033,086, incorporated by reference), using the protocol illustrated in Figure 9A, with the following modifications. Specifically, two synthetic adaptors, instead of one, were 30 used for ligation with DNAase I-generated cDNA fragments. One adaptor, containing three ATG codons, carried a *HindIII* cloning site (Figure 9B). The other adaptor had translation stop codons in all three reading frames and carried a *Clal*

- 23 -

cloning site (Figure 9B). After ligation with the equimolar mixture of both adaptors, cDNA fragments were amplified by PCR using sense and antisense strands of the first and second adaptor, respectively. PCR products were digested with *Clal* and *HindIII* and cloned into the corresponding sites of the pLNCX plasmid. This
5 modification of the cloning strategy resulted in avoiding the formation of inverted repeats at the ends of the cDNA inserts after cloning into the retroviral vector.

A plasmid library of 20,000 independent insert-carrying clones was obtained and transfected into ecotropic packaging cells using the calcium phosphate precipitation technique. Virus released by transiently transfected cells was used to
10 infect HT1080/pJET-2TGH cells, clone 2, a derivative of human HT1080 fibrosarcoma cell line transfected with a plasmid expressing the murine ecotropic receptor (Albritton *et al.*, 1989, Cell 57: 659-666) and susceptible to infection with ecotropic retroviruses (provided by Dr. G.R. Stark, Cleveland Clinic Foundation). After infection and G418 selection, these cells (further referred to as HT1080/ER)
15 were plated at 10^5 cells per 100 mm plate and cultivated for 12 days in different concentrations of etoposide (200-500 ng/mL). After removal of the drug, cells were allowed to grow in media without drug for 7 more days. At this point, some of the plates were fixed and stained with crystal violet, to determine the number of surviving colonies (Figure 10). As illustrated in Figure 10, at drug concentrations
20 of 250 ng/mL etoposide, there were only several colonies in control plates, compared with about a hundred times more colonies in the plates containing GSE-containing cells.

In a parallel experiment, virus-producing mixtures of packaging cells were subjected to similar etoposide selection. At all drug concentrations tested, there were
25 many more colonies surviving etoposide treatment in the GSE-carrying cells than in the control cell population.

These results indicated that the retroviral library of random fragments of KHCS cDNA contained numerous GSEs inducing drug resistance in human cells, confirming that human KHCS is associated with drug resistance. Some of these
30 GSEs are likely to be more potent as selectable markers of drug resistance than the original single GSE from the murine *khcs* gene. The virus isolated from such etoposide-resistant cells represents a collection of a multiplicity of kinesin-derived,

- 24 -

drug resistance-conferring GSEs, which multiplicity is itself an aspect of the present invention and is useful in conferring resistance to DNA damaging agents, including chemotherapeutic drugs, as disclosed herein.

5

EXAMPLE 7**Generation of Cell Populations Carrying Multiple Copies of anti-*khcs* GSEs**

A 1:1 mixture of ecotropic and amphotropic packaging cells was transfected with retroviral vector pLNCX carrying the anti-*khcs* GSE using a standard calcium phosphate procedure. Two weeks later, the virus titer, as measured by the formation of G418-resistant NIH 3T3 colonies, reached $>10^6$ infectious units per mL as a result of "ping-pong" infection (*see* Bodine *et al.*, 1990, Proc. Natl. Acad. Sci. USA 87: 3738-3742). This virus-containing supernatant was used to infect NIH 3T3 cells, 10 times with 12 hour intervals. Control cells were infected in parallel with the insert-free vector virus obtained by the same procedure. G418 selection showed that 100% of NIH 3T3 cells became infected with the virus. DNA from the infected cells was analyzed by Southern blot hybridization with a virus-specific probe. This analysis showed that the infected cells contained multiple copies of the integrated provirus.

Freshly-obtained multiply-infected NIH 3T3 cells were characterized by a decreased growth rate and plating efficiency. After several passages, however, their growth parameters became indistinguishable from the control cells, suggesting the elimination of slowly growing cells from the population. At this stage, the cells were frozen and used for the experiments described below.

25

EXAMPLE 8**Drug Resistance Pattern of NIH 3T3 Cells Carrying Multiple Copies of Anti-*khcs* GSEs**

The infected cell populations described in Example 7 were analyzed for resistance to several anticancer drugs by a growth inhibition assay. For this assay, 10^4 cells per well were plated in 12-well plates and exposed to increasing concentrations of different drugs for 4 days. Relative cell numbers were measured by the methylene blue staining assay (Perry *et al.*, 1992, Mutation Res. 276: 189-197). Despite the relative insensitivity of this assay when carried out with unselected

- 25 -

heterogeneous cell populations, infection with the virus carrying an anti-*khcs* GSE induced a pronounced increase in the resistance to the cytostatic effects of etoposide and amsacrine and, to a lesser extent, of Adriamycin, camptothecin and cisplatin (Figure 11). All of these drugs are known to induce DNA damage, albeit by different mechanisms. Under the same assay conditions, no increase in resistance was observed with colchicine or actinomycin D (Figure 11).

To further characterize the nature of drug resistance conferred by anti-*khcs*, the above-described short-term growth inhibition assays were followed by long-term growth inhibition assays which measured both the cytostatic and the cytotoxic effects of different drugs. These assays were carried out by incubating the cells for four days in the presence of the drugs, followed by either two or six days in the absence of the drug, to allow for programmed cell death, which is frequently associated with recovery from drug-induced inhibition (Kung *et al.*, 1990, Cancer Res. 50: 7307-7317). These assays showed that the GSE-carrying cells were resistant to etoposide and adriamycin, but not to cisplatin, camptothecin or actinomycin D. Furthermore, the GSE-carrying cells were found to be hypersensitive to colchicine and vinblastine, said hypersensitivity being increasingly more evident with increasing length of the assay.

Thus, in the experiment shown in Figure 12, NIH 3T3 cells carrying the anti-*khcs* GSE and control cells without the GSE were plated at a density of 2×10^4 per culture dish and grown for five days in concentrations of either colchicine or vinblastine. The cells were then fixed and stained, and the number of surviving cells determined and shown as a percentage of cell growth in the absence of drug. These results clearly show that expression of the anti-*khcs* GSE in these cells was accompanied by hypersensitivity to both colchicine and vinblastine.

To further investigate the discrepancy between the results obtained in the short-term and long-term drug assays, analyses of the dynamics of cell growth during and after treatment with 250 ng/mL etoposide, 20 ng/mL camptothecin and 40 ng/mL colchicine were performed. In these experiments (shown in Figure 13), NIH 3T3 cells were treated with the corresponding drugs for 5 days, and then incubated for additional 6 days either in the presence or in the absence of the drug. The selected drug concentrations resulted in growth inhibition but little detectable cell

- 26 -

death in continuous presence of the drug. After 11 days of drug exposure the total cell number in the etoposide- or camptothecin-treated populations of control cells was a little lower than after 5 days of drug exposure, but in the colchicine-treated control cell population a slow cell growth was still detectable. In the experiments where the drug was removed after 5 days, cell growth was initially activated for all three drugs. After about two days of growth in the absence of the drug, the number of cells treated with etoposide or camptothecin decreased, however, due to extensive cell death, so that the final cell number in cell populations incubated in the absence of the drugs was practically the same as in the cells continuously maintained in the presence of colchicine or camptothecin. In contrast, cells pre-treated with colchicine undergo only limited cell death after removal from the drug, resulting in a relatively minor slowdown in cell growth two days after removal from the drug, and a major increase in the total cell number in the populations removed from the drug relative to those that were constantly maintained in colchicine (Figure 13).

The expression of the anti-*khcs* GSE had different effects on the growth inhibition and recovery-associated cell death induced by these drugs. In cells treated with etoposide, anti-*khcs* decreased the growth-inhibitory effect of the drug to the same, relatively small extent after 5 or 11 days of continuous exposure. In addition, the anti-*khcs* GSE decreased the amount of cell death in the populations released from etoposide inhibition. As a result, the increase in etoposide resistance conferred by this GSE was much more pronounced after release from the drug than under the conditions of continuous exposure (Figure 13). In cells treated with camptothecin, the GSE resulted in a small decrease of the growth-inhibitory effect of the drug, which was more pronounced after 5 days of exposure but was reduced to negligible levels after 11 days of continuous exposure. The decrease in growth inhibition after 5 days of exposure was accompanied by a slight increase in cell death after the removal from the drug, so that the GSE produced no significant long-term difference in camptothecin resistance. In the populations treated with colchicine, the anti-*khcs* GSE made cells more susceptible to the growth-inhibitory effect of the drug; this effect was increased with prolonged exposure and was equally apparent in the populations released from the drug after 5 days or continuously maintained in colchicine (Figure 13).

- 27 -

The results of the above experiments indicated that the anti-*khcs* GSE acts by inhibiting the cytostatic effects of different DNA-damaging drugs. In addition, this GSE appears to decrease the extent of programmed cell death occurring after the release from the drug in cells treated with some (etoposide) but not other
5 (camptothecin) DNA-damaging agents.

The finding that cells carrying the anti-*khcs* GSE become hypersensitive to the cytostatic effects of colchicine has potentially significant therapeutic implications. The observed hypersensitivity to colchicine, an anti-microtubular agent, in cells with GSE-mediated inhibition of kinesin is likely to be mechanistically related to the
10 essential functions of kinesin, which moves various structures along the microtubules and may be involved in the sliding of microtubules relative to each other, as well as the assembly and disassembly of microtubules. The pronounced effect of the anti-*khcs* GSE on the sensitivity of cells to anti-microtubule agents also indicates that this GSE affects the general kinesin function in the cells, and is not limited to a particular
15 drug-response-specific isoform of kinesin. Since we have demonstrated that down-regulation of the *KHCS* gene represents a natural mechanism of drug resistance in human tumor cells (*see* Example 11), the hypersensitivity to colchicine provides an approach to overcoming this type of resistance in human cancer.

20

EXAMPLE 9

Assessment of Cellular Effects Of Anti-kinesin GSEs

The virus carrying the anti-*khcs* GSE was tested for the ability to increase the life span of primary mouse embryo fibroblasts (MEF). MEF were prepared from 10 day old mouse embryos by a standard trypsinization procedure and senescent cells
25 were frozen at different passages prior to crisis. Senescent MEF, two weeks before crisis, were infected with recombinant retroviruses carrying LNCX vector either without an insert or with anti-*khcs*. Figure 14 shows MEF cell colonies two weeks after crisis. Relative to uninfected MEF cells, or cells infected with a control LNCX virus, cells infected with the anti-*khcs* showed a great increase in the proportion of
30 cells surviving the crisis. Post-crisis cells infected with the anti-*khcs* virus showed no microscopically visible features of neoplastic transformation. These results indicate that anti-*khcs* promotes the immortalization of normal senescent fibroblasts.

- 28 -

These results suggest that the normal function of kinesin-S may be associated with the induction of programmed cell death occurring after exposure to certain cytotoxic drugs or in the course of cellular senescence. These results also indicate that isolation of GSEs that confer resistance to chemotherapeutic drugs can provide
5 insight into the cellular genes and processes involved in cell growth regulation.

EXAMPLE 10

Biological Effects of Mouse Anti-*khcs* GSE on Human Senescent Fibroblasts

The ability of the anti-*khcs* GSE described in Example 4 to promote
10 immortalization of primary mouse embryo fibroblasts (demonstrated in Example 9) suggested that kinesin-S may act as a tumor suppressor by preventing immortalization of normal mouse fibroblasts. To determine if this gene may play the same role in human cells, the ability of the anti-*khcs* GSE to affect the life span of primary human fibroblasts was investigated.

15 Primary human fibroblasts, derived from human skin, were obtained from the Aging Cell Repository of the National Institutes of Aging. The cells were grown in DMEM with 20% fetal calf serum supplemented with twice the concentration of amino acids and vitamins normally used to supplement normal human skin fibroblast growth in culture. Cells at the fifth passage were infected with either the control
20 pLNCX virus or the virus carrying mouse anti-*khcs* GSE (produced as described above in Example 3). Four passages later, the control cells went into crisis, but GSE-carrying cells continued to grow (Figure 15) and have so far survived at least five additional passages. These results demonstrated that the anti-*khcs* GSE of this invention is also capable of prolonging life span of primary human fibroblasts,
25 indicating that *KHCS* is a potential tumor suppressor in human cells.

EXAMPLE 11

Assessment Of The Role Of Decreased *khcs* Gene Expression In Naturally Occurring Mechanisms Of Drug Resistance

30 To test whether decreased *khcs* gene expression is associated with any naturally occurring mechanisms of drug resistance, an assay was developed for measuring *khcs* mRNA levels by cDNA-PCR. This assay is a modification of the

- 29 -

quantitative assay described by Noonan *et al.* (1990, Proc. Natl. Acad. Sci. USA 87: 7160-7164) for determining *mdr-1* gene expression. The oligonucleotide primers had the sequences:

AGTGGCTGGAAAACGAGCTA (SEQ.ID.No.:5) and
5 CTTGATCCCTTCTGGTTGAT (SEQ.ID.No.:6).

These primers were used to amplify a 327 bp segment of mouse *khcs* cDNA, corresponding to the anti-*khcs* GSE. These primers efficiently amplified the mouse cDNA template but not the genomic DNA, indicating that they spanned at least one intron in the genomic DNA. Using these primers, we determined that *khcs* mRNA
10 is expressed at a higher level in the mouse muscle tissue than in the kidney, liver or spleen.

In another experiment a pair of primers amplifying a homologous segment of the human KHCS cDNA was selected, based on the reported human KHC sequence published by Navone *et al.* (1992, J. Cell. Biol. 117: 1263-1275). The sequences
15 of these primers are:

AGTGGCTTGAAAATGAGCTC (SEQ.ID.No.:7) and
CTTGATCCCTTCTGGTAGATG (SEQ.ID.No.:8),

and they amplify a 327 bp cDNA fragment. These primers were used to test for changes in the KHCS gene expression in several independently isolated populations
20 of human HeLa cells, each selected for spontaneously acquired etoposide resistance; β_2 -microglobulin cDNA sequences were amplified as an internal control. Figure 16 shows the results of the cDNA-PCR assay on the following populations: CX(0), HeLa population infected with the LNCX vector virus and selected with G418; CX (200), the same cells selected for resistance to 200 ng/ml etoposide; E11(O), 6(O)
25 and E21(O), populations obtained after infection of HeLa cells with recombinant retroviruses carrying different GSEs derived from topoisomerase α cDNA, as described in Example 1 of co-pending U.S. Patent Application Serial No. 08/033,086, incorporated by reference, and selected with G418; E11 (1000), 6(1000) and E21(1000), the same populations selected for resistance to 1 μ g/ml etoposide.
30 As shown in Figure 16, the yield of the PCR product specific for the *khcs* gene was significantly lower in each of the etoposide-selected populations than in the control

- 30 -

cells. This result indicates that a decrease in the *khcs* gene expression is a common natural mechanism for drug resistance.

EXAMPLE 12

Diagnostic Assay

5

The results presented in the above Examples suggest the utility of diagnostic assays for determining the expression levels of kinesin genes in tumor cells of a cancer patient, relative to a standardized set of cell lines *in vitro* having well-characterized levels of kinesin heavy chain gene expression correlated with their level of resistance to certain chemotherapeutic drugs such as etoposide. One such standardized set of cell lines comprise the HeLa cell lines described in Example 11. Alternatively, different, tissue-specific standardized sets of cell lines are developed by drug selection for each cell type to be evaluated, for example, using human K562 cells for evaluating patients having chronic myelogenous leukemia, or human HL60 cells for patients having acute promyelocytic leukemia.

15

The assay for kinesin would assess the appropriateness of treatment of human cancer patients with certain anticancer therapeutic regimens. Patients whose tumor cells under-express kinesin may be refractory to treatment with DNA damaging agents, including radiation and the chemotherapeutic drugs etoposide, camptothecin, cisplatin and adriamycin. Such patient, however, may be particularly responsive to treatment with anti-microtubular agents such as colchicine, colcemide, vinblastine, vincristine or vindesine. On the other hand, patients whose tumor cells over-express kinesin, for example, may be responsive to treatment with DNA damaging agents and refractory to treatment with anti-microtubular agents. These assays provide, for the first time, a basis for making such therapeutic judgments before the fact, rather than after a therapeutic regimen has been tried and failed. The assay also provide a basis for determining which patients, previously refractory to treatment with DNA damaging agents, particularly certain anticancer drugs, would benefit from further chemotherapy using anti-microtubular agents, by distinguishing kinesin gene-mediated drug resistance from other mechanisms of drug resistance expected to result in cross-resistance to both DNA damaging agents and anti-microtubular drugs.

20

25

30

- 31 -

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

- 32 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Board of Trustees of the University of Illinois
- (B) STREET: 352 Henry Administration Building,
506 South Wright Street
- (C) CITY: Urbana
- (D) STATE: Illinois
- (E) COUNTRY: USA
- (F) POSTAL CODE (ZIP): 61801
- (G) TELEPHONE:
- (H) TELEFAX:

(ii) TITLE OF INVENTION: Association of Kinesin with Sensitivity
To Chemotherapeutic Drugs

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AATCATCGAT GGATGGATGG

20

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

- 33 -

CCATCCATCC ATCGATGATT AAA

23

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 327 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTTGATCCCT TCTGGTTGAT GCCAGAAGCT CTTCTGATC CAGCATTGT ATCTTCAATT	60
TCTCTACCAA TTGGCTTTGT TGGTTAATCT CTTCATCCTT GTCATCAAGT TGTTTATACA	120
ATTTAGCAAG TTCTTCTTCA CACTTCTTC TTTGAGCATC GGTAAACTA CCAGCCATTC	180
CGACTGCAGC AGCTGGTTTA TCACTGGTAA TAGCAATATC TTTATCCGCT GTGAAGGCTT	240
CCAAATTAGC TTTCTCTTG TCAAACTGCT CATCAATAGG CACTGTCTCC CCGTTACGCC	300
AACGGTTTAG CTCGTTTCC AGCCACT	327

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2389 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGACAAACAT CATCTGGGAA GACCCACACG ATGGAGGGTA AACTTCATGA TCCAGAAGGC	60
ATGGGAATTA TTCCAAGAAT AGTGCAAGAT ATTTTAAATT ATATTTACTC CATGGATGAA	120
AATTTGGAAT TTCATATTAA GGTTCATAT TTTGAAATAT ATTTGGATAA GATAAGGGAC	180
TTGTTAGATG TTTCAAAGAC TAACCTTTC GTCCATGAAG ACAAAAACCG TGTTCCCTAT	240
GTAAAGGGGT GCACAGAACG TTTCGTGTGT AGTCCAGATG AAGTCATGGA TACCATAGAT	300
GAAGGGAAT CCAACAGAGA TGTCGCAGTT ACAAATATGA ATGAACATAG CTCTAGGAGC	360
CACAGCATAT TTCTTATTAA TGTAACAAC GAGAATACAC AAACGGAACA GAAACTCAGT	420
GGAAAGCTTT ATCTGGTTGA TTTAGCTGGC AGTGAGAAGG TTAGTAAGAC TGGGGCTGAA	480
GGTGCTGTGC TGGATGAAGC TAAGAACATC AAGAAGTCAC TTTCTGCACT TGGAAATGTC	540

- 34 -

ATTTCTGCTT TGGCAGAGGG CAGTACCTAT GTTCCTTATC GAGATAGTAA AATGACCAGA	600
ATTCTTCAAG ATTCATTAGG TGGCAACTGT AGGACCACTA TTGTCATATG CTGCTCTCCA	660
TCATCATACA ATGAGTCTGA GACAAAGTCA ACACCTCTCT TTGGTCAAAG GGCCAAAACA	720
ATTAAGAACA CAGTCTGTGT CAATGTAGAG TTAAGTGCAG AGCAGTGGAA AAAGAAGTAT	780
GAAAAAGAAA AGGAAAAAAA TAAGACTCTA CGGAACACTA TTCAGTGGCT GGAAAAACGAG	840
CTAAACCGTT GGCCTAACGG GGAGACAGTG CCTATTGATG AGCAGTTTGA CAAAGAGAAA	900
GCTAATTTGG AAGCCTTCAC AGCGGATAAA GATACTGCTA TTACCAGTGA TAAACCAGCT	960
GCTGCAGTCG GAATGGCTGG TAGTTTTACC GATGCTGAAA GAAGAAAGTG TGAAGAAGAA	1020
CTTGCTAAAT TGTATAAACA GCTTGATGAC AAGGATGAAG AGATTAACCA ACAAGCCAA	1080
TTGGTAGAGA AATTGAAGAC ACAAATGCTG GATCAGGAAG AGCTTCTGGC ATCAACCAGA	1140
AGGGATCAAG ATAATATGCA AGCTGAACTG AATCGCCTCC AAGCAGAAAA TGATGCTTCT	1200
AAAGAAGAAG TCAAAGAAGT TTTACAGGCC TTAGAGGAAC TGGCTGTAA TTATGATCAG	1260
AAGTCTCAGG AAGTTGAAGA CAAAACAAAG GAATATGAAT TGCTTAGTGA TGAATTGAAT	1320
CAAAAATCTG CAACCTTAGC AAGTATTGAT GCTGAGCTTC AGAAGCTGAA GGAAATGACC	1380
AACCACCAGA AGAAACGAGC AGCTGAAATG ATGGCATCAT TATTAAGA CTTGCAGAA	1440
ATAGGAATTG CTGTGGGGAA TAACGATGTG AAGCAACCAG AAGGAACTGG TATGATAGAT	1500
GAAGAGTTTA CTGTTGCAAG ACTCTACATT AGCAAAATGA AATCAGAAGT AAAGACCATG	1560
GTGAAACGCT GCAAACAGCT AGAAAGCAGC CAGACTGAGA GCAACAAAAA AATGGAAGAA	1620
AATGAGAAAG AGTTAGCAGC ATGCCAGCTT CGGATCTCCC AACATGAAGC CAAAATCAAG	1680
TCACTGACTG AGTACCTTCA GAATGTAGAA CAAAAGAAGA GGCAGCTGGA GGAATCTGTT	1740
GATTCCCTTG GTGAGGAGCT AGTCCAATC CGAGCACAAG AGAAAGTCCA TGAAATGGAA	1800
AAAGAGCACT TGAACAAGGT TCAGACTGCA AATGAAGTCA AGCAAGCTGT TGAGCAGCAG	1860
ATCCAGAGTC ACAGAGAAAC CCACCAAAAA CAAATCAGTA GCTTGCGAGA TGAAGTTGAG	1920
GCAAAGGAAA AGCTAATCAC TGACCTCAA GACCAAAACC AGAAGATGGT GTTGAGCAG	1980
GAACGGCTAA GGGTGGAGCA TGAGAGGCTG AAGGCTACAG ACCAAGAGAA GAGCAGGAAG	2040
CTGCATGAGC TCACGGTTAT GCAAGACAGA CGAGAACAAG CAAGACAAGA CTTGAAGGGT	2100
TTGGAGGAGA CCGTGGCAAA AGAACTTCAG ACTTTACACA ACCTGCGTAA GCTCTTTGTT	2160
CAGGACTTGG CTACCAGGGT GAAAAAGAGG CCGAGGTCGA CTCTGACGAC ACTGGCGGCA	2220
GTGCTGCACA GAAGCAGAAA ATCTCCTTCC TTGAAAACAA CTTGAACAG CTCACCAAAG	2280
TGCACAAGCA GTTGGTACGT GATAATGCAG ATCTTCGCTG TGAGCTTCCT AAGTTAGAGA	2340
AACGGCTTAG AGCTACTGCA GAAAGAGTGA AAGCTTTGGA GTCAGCCCCG	2389

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid

- 35 -

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTCCCAAGCT TATGGATGGA TG

22

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CATCCATCCA TAAGCTTGGG AGAAA

25

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TGAGTGAGTG AATCGATGAT TAAA

24

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

- 36 -

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AATCATCGAT TCACTCACTC A

21

WE CLAIM:

1. A method of identifying genetic suppressor elements derived from a kinesin gene that confer upon a cell resistance to one or more DNA damaging agents, the method comprising the steps of:

- 5 (a) generating a set of random fragments of cDNA encoding the kinesin gene;
- (b) transferring the DNA fragments to an expression vector to yield a library, wherein the expression vector is capable of expressing the DNA fragments in a living cell;
- 10 (c) genetically modifying living cells by introducing the random fragment library of step (b) into the living cells;
- (d) isolating or enriching for genetically modified living cells containing kinesin-derived genetic suppressor elements by selecting cells in the presence of a DNA damaging agent; and
- 15 (e) obtaining the genetic suppressor element from the genetically modified cells.

2. A genetic suppressor element identified by the method of claim 1.

3. A genetic suppressor element according to claim 2 having a nucleotide sequence that is homologous to a portion of the human or mouse *khcs* gene.

20 4. A mammalian cell that expresses a GSE according to claim 2.

5. A mammalian cell that expresses a GSE according to claim 3.

6. The method of Claim 1, wherein the genetic suppressor element is sense-oriented.

25 7. The method of Claim 1, wherein the genetic suppressor element is antisense-oriented.

8. A synthetic peptide having an amino acid sequence corresponding to from about 6 amino acids to all of the amino acid sequence encoded by the GSE produced according to the method of Claim 6.

9. A synthetic oligonucleotide having a nucleotide sequence from about 12 nucleotides to all of the nucleotide sequence of the antisense RNA encoded by the GSE produced by Claim 7.

10. A diagnostic assay comprising the steps of

(a) isolating cellular RNA comprising messenger RNA from a tumor or malignant cells from an animal;

(b) measuring a level of expression of kinesin mRNA in the tumor or malignant cells in the animal;

(c) determining whether the level of expression of kinesin mRNA in the tumor or malignant cells indicates that the kinesin gene is over-expressed or under-expressed in the tumor or malignant cells in the animal.

11. A diagnostic assay comprising the steps of

(a) isolating cellular protein from a tumor or malignant cells from an animal;

(b) measuring an amount of a kinesin protein in the tumor or malignant cells in the animal;

(c) determining whether the amount of the kinesin protein in the tumor or malignant cells indicates that the kinesin gene is over-expressed or under-expressed in the tumor or malignant cells in the animal.

12. A method of treating an animal having a malignant tumor or malignant cells in the animal's body, the method comprising administering an anticancer drug that is a DNA damaging agent to the animal if the tumor or malignant cells over-express a kinesin gene as determined using the assay of Claims 10 or 11.

13. A method of treating an animal having a malignant tumor or malignant cells in the animal's body, the method comprising administering an anticancer drug that is an anti-microtubule agent to the animal if the tumor or malignant cells under-express a kinesin gene as determined using the assay of Claims 10 or 11.

5 14. A method of treating an animal having a malignant tumor or malignant cells in the animal's body, the method comprising administering an anticancer drug that is an anti-microtubule agent to the animal if the tumor or malignant cells under-express a kinesin gene as determined using the assay of Claims 10 or 11 and the animal was previously unsuccessfully treated with an anticancer drug that is a DNA
10 damaging agent.

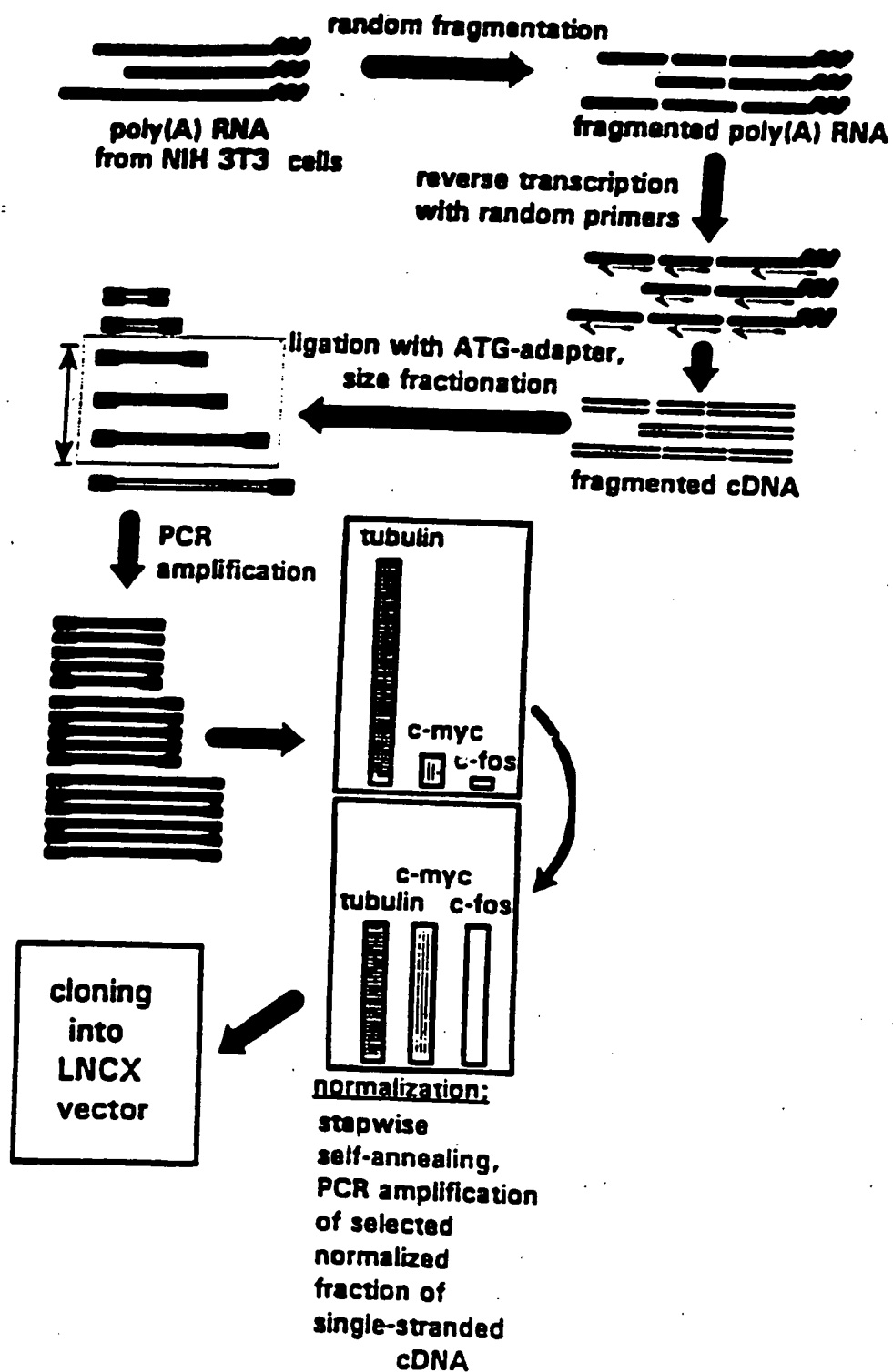
15. A method of identifying a multiplicity of genetic suppressor elements derived from a kinesin gene, that confer upon a cell resistance to one or more DNA damaging agents, the method comprising the steps of:

- 15 (a) generating a set of random fragments of cDNA encoding the kinesin gene;
- (b) transferring the DNA fragments to a retroviral expression vector to yield a retroviral random fragment expression library, wherein the retroviral expression vector is capable of expressing the DNA fragments in a living cell;
- 20 (c) genetically modifying a culture of living cells by introducing the retroviral random fragment expression library of step (b) into the living cells of the culture, wherein the living cells of the culture are capable of propagating each of the retroviral expression vectors comprising the library;
- 25 (d) isolating or enriching for genetically modified living cells containing kinesin-derived genetic suppressor elements by selecting cells in the presence of a DNA damaging agent; and
- (e) allowing the drug-resistant, genetically modified living cells of step (d) to propagate the kinesin-derived genetic suppressor elements of the
30 retroviral random fragment expression library to form an amplified

- 40 -

random fragment expression library comprising a multiplicity of kinesin-derived genetic suppressor elements.

16. A genetic suppressor element first identified by the method of claim 15.
17. A genetic suppressor element according to Claim 16 having a nucleotide
5 sequence that is homologous to a portion of the human or mouse *khcs* gene.
18. A mammalian cell that expresses a GSE according to claim 16.
19. A mammalian cell that expresses a GSE according to claim 17.
20. The method of Claim 15, wherein the genetic suppressor element is
10 sense-oriented.
21. The method of Claim 15, wherein the genetic suppressor element is antisense-oriented.
22. A synthetic peptide having an amino acid sequence corresponding to
from about 6 amino acids to all of the amino acid sequence encoded by the GSE
15 produced according to the method of Claim 21.
23. A synthetic oligonucleotide having a nucleotide sequence from about
12 nucleotides to all of the nucleotide sequence of the antisense RNA encoded by the
GSE produced by Claim 22.

Figure 1A

2 / 3 4

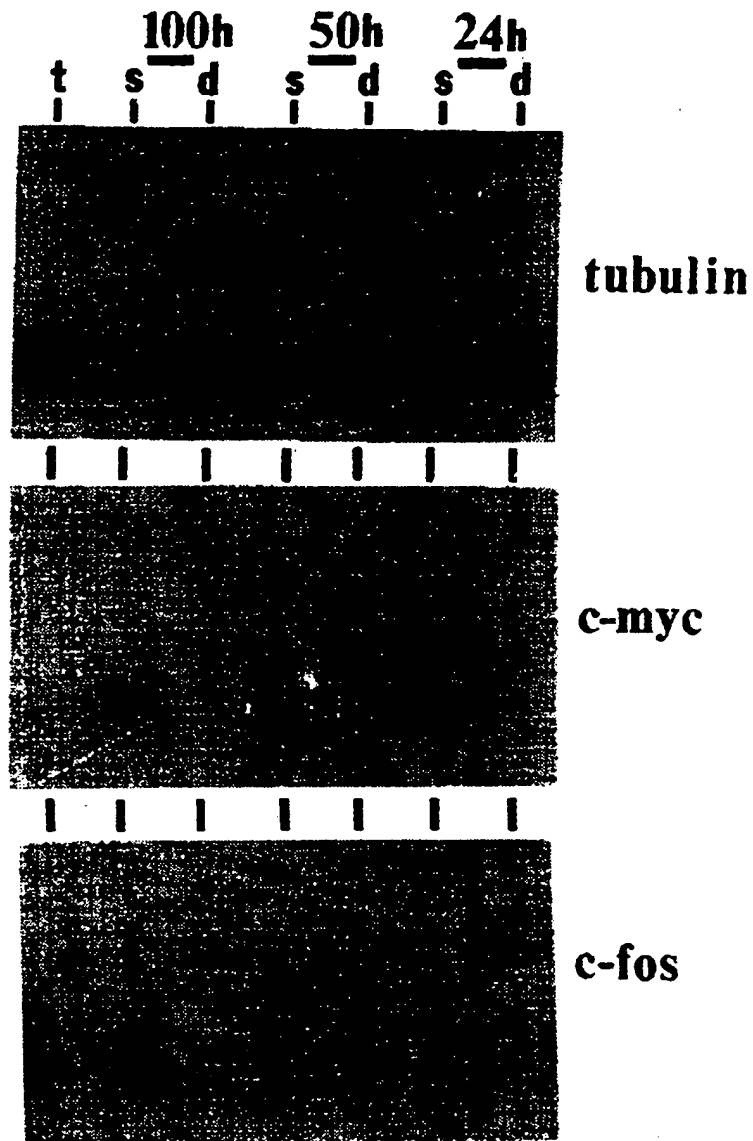
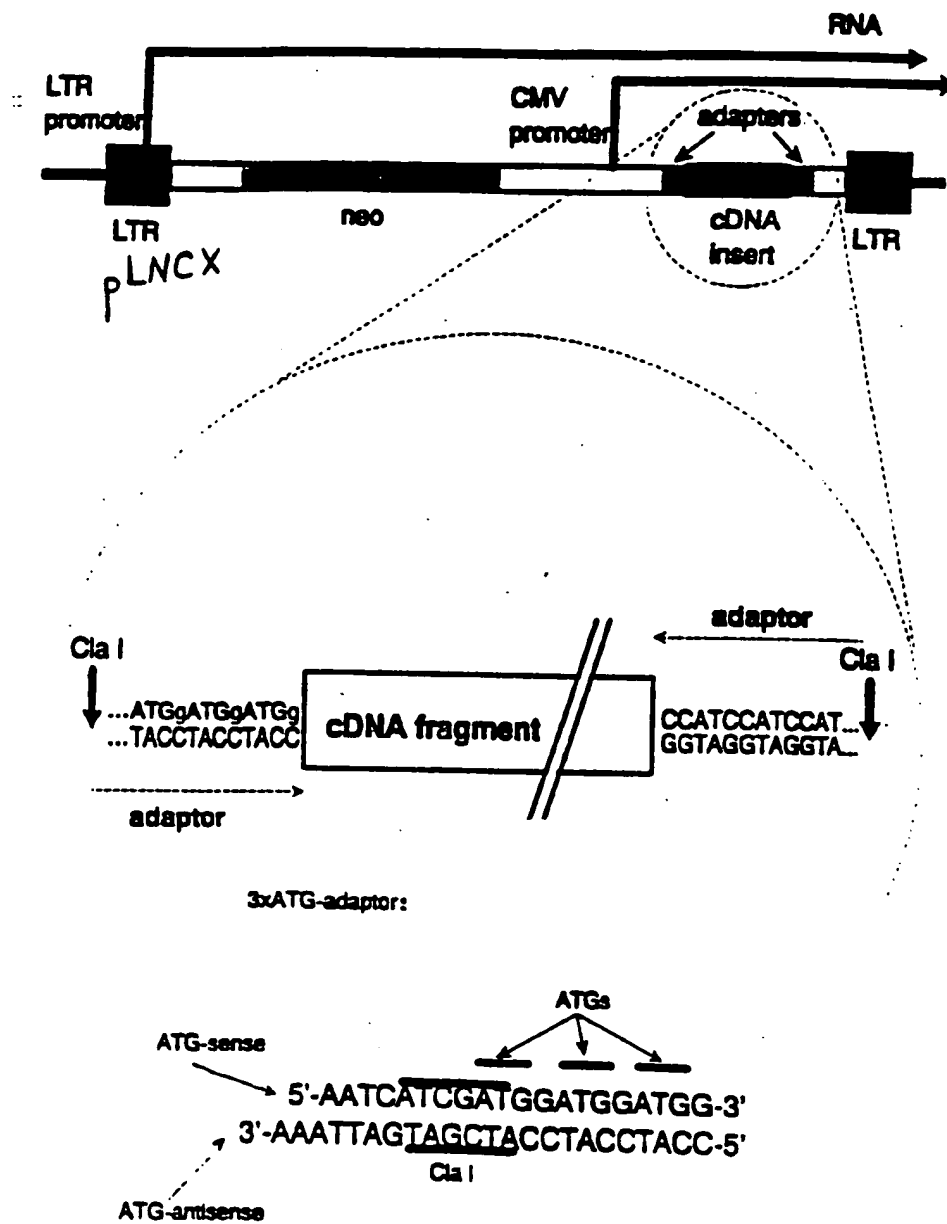
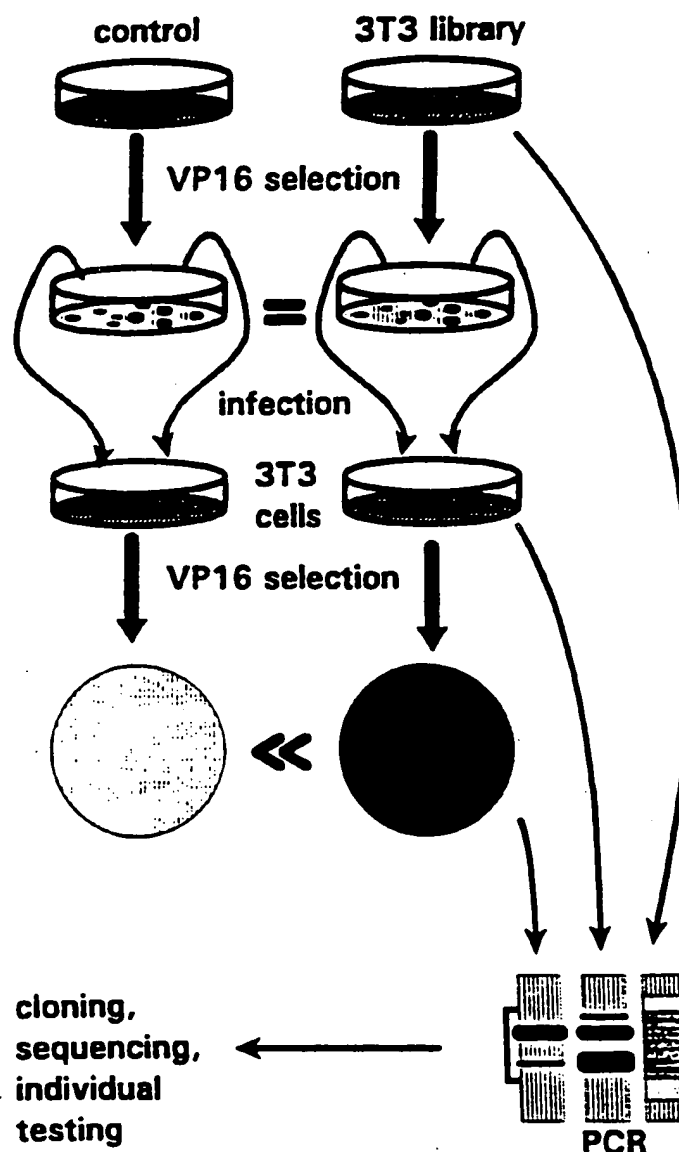
Figure 1B

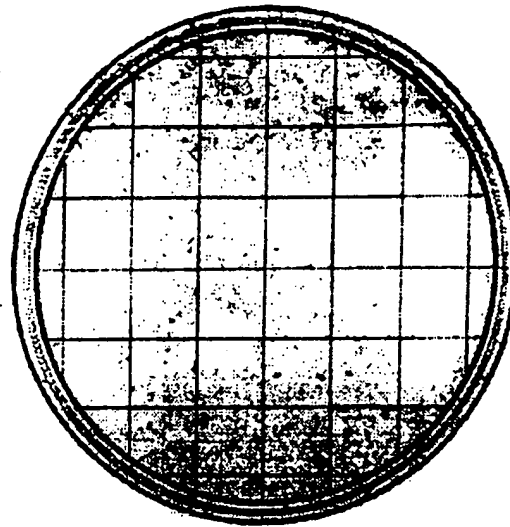
Figure 2

4 / 3 4

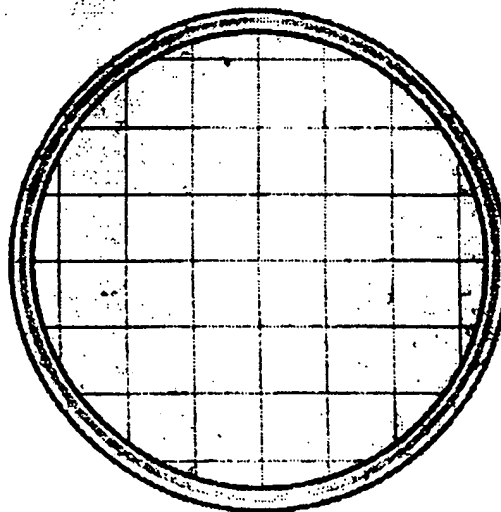
Figure 3

5 / 3 4

Figure 4A



infection



control

6 / 3 4

Figure 4B

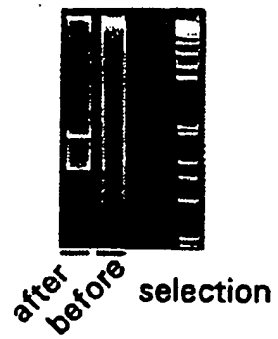


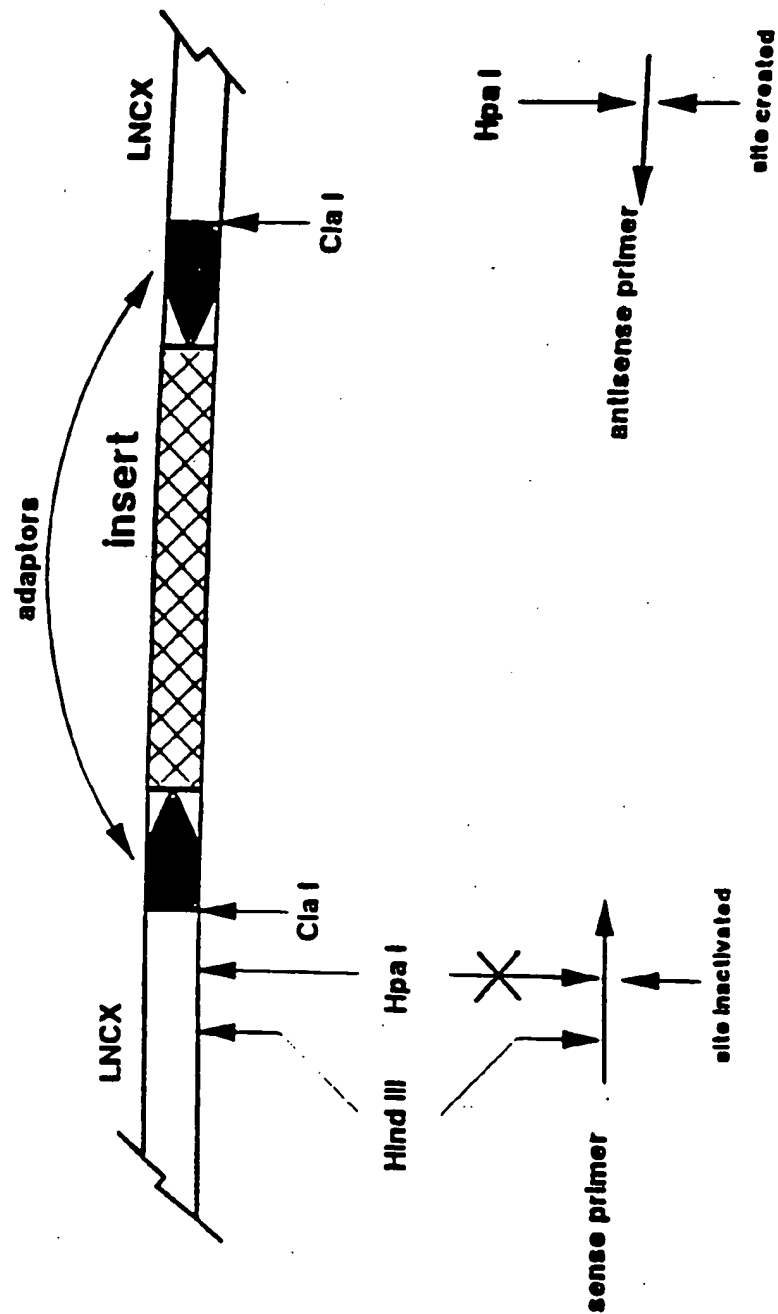
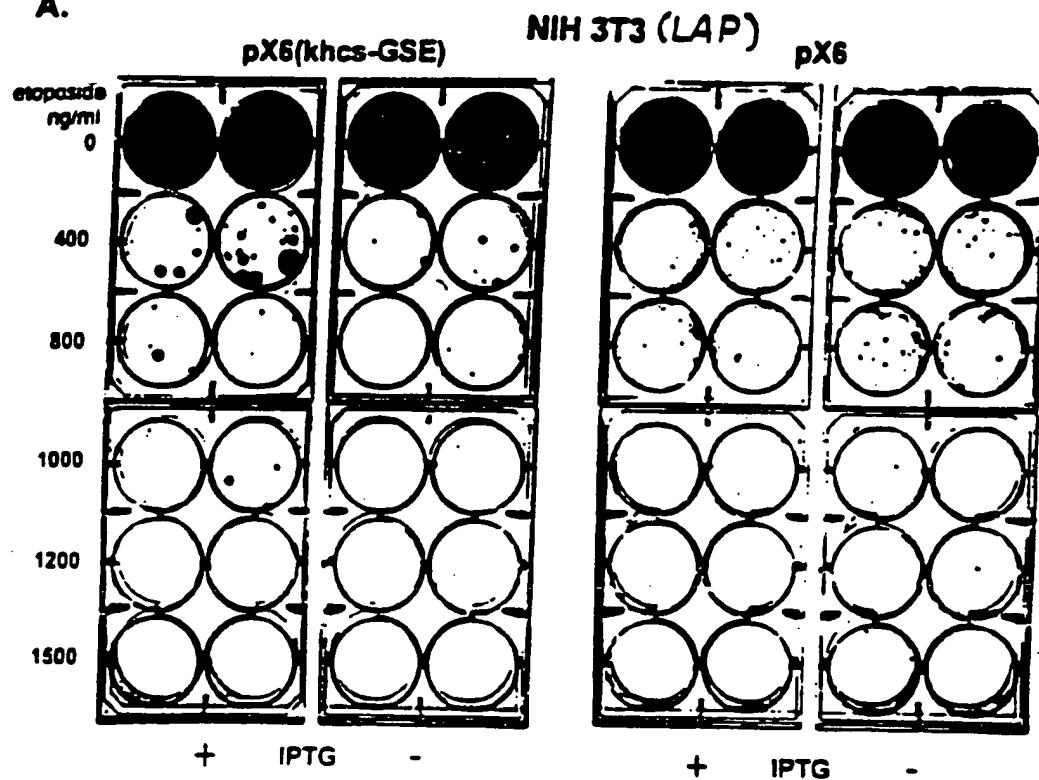
Figure 4C

Figure 5A

Biological effects of khc-s GSE isolated from a normalized NIH 3T3 cDNA RFL

A.



9 / 3 4
Figure 5B

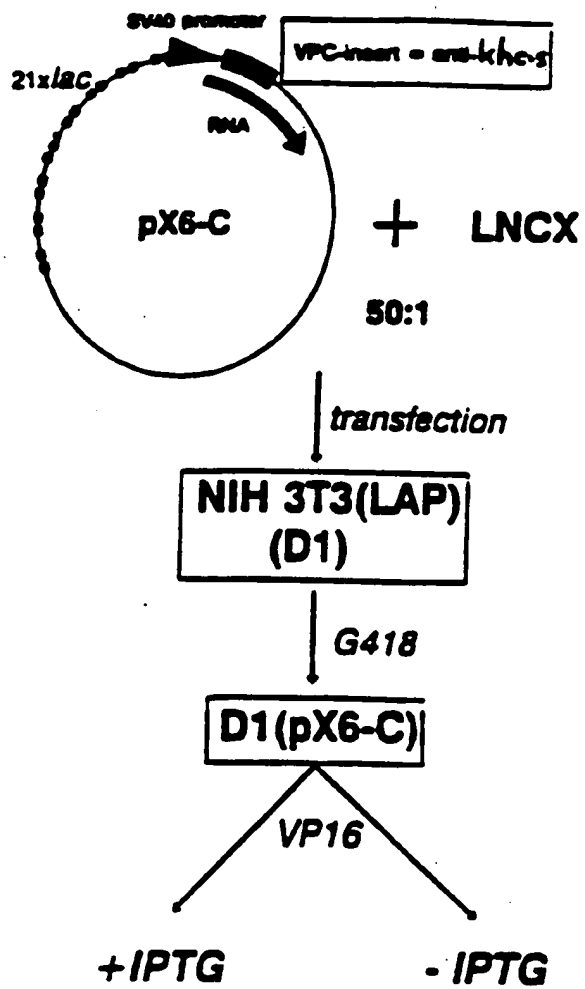


Figure 6

GSE VPC (anti khc-s)

CTTGATCCCT	TCTGGTTGAT	GCCAGAAAGCT	CTTCCTGATC	CAGCATTTGT	ATCTTCAATT	60
TCTTACCAA	TTGGCTTTGT	TGGTAAATCT	CTTCATCCCT	GTCAATCAAGT	TGTTTATACA	120
ATTAGCAAG	TTCTTCTTCA	CACTTCTTC	TTTCAGCATC	GGTAAACTA	CCAGCCATTG	180
CGACTGCAGC	AGCTGGTTTA	TCACTGGTAA	TAGCAATATC	TTTATCCGCT	GTGAGGCTT	240
CCAAATTAGC	TTCTCTTTTG	TCAAACTGCT	CATCAATAGG	CACTGTCTCC	CCGTTACGCC	300
AACGGTTAG	CTCGTTTTC	AGCCACT				327

Figure 7

CGACAAACAT CATCTGGGAA GACCCACACG ATGGAGGGTA AACTTCATGA TCCAGAAGGC 60
 ATGGGAATTA TTCCAAGAA AGTGCAGAT ATTTTAAT ATATTACTC CATGGATGAA 120
 AATTTGGAAT TTCAATATA GTTTTCATAT TTGAATAT ATTTGATAA GATAAGGGAC 180
 TTGTTAGATG TTCAAGAGAC TAACCTTTCA GTCCATGAAG ACATAAACCG TGTTCCCTAT 240
 GTAAAGGGGT GCACAGAAGG TTTGCTGTGT AGTCCAGATG AAGTCATGGA TACCATAGAT 300
 GAAGGGAAT CCAACAGAGA TGTCCGAGTT ACAATATGA ATGAACATAG CTCTAGGAGC 360
 CACAGCATAT TTCTTATTA TGTAAACAA GAGATACAC AACCGAACA GAAACTCAOT 420
 GGAAGCTTT ATCTGTTGA TTTAGCTGGC AGTGAGAAGG TTAGTAAGAC TGGGGCTGAA 480
 GTGCTGTGC TGGATGAAGC TAAGAACATC AAGTGAAGG TTAGTAAGAC TGGGGCTGAA 540
 ATTTCTGCTT TGGCAGAGGG CAGTACCTAT GTTCTTATC GAGATAGTAA AATGACCAGA 600
 ATTCTTCAAG ATTCATTAGG TGGCAACTGT AGGACCACTA TTGTCATATG CTGCTCTCCA 660
 TCATCATACA ATGAGTCTGA GACAAAGTCA ACATCTCTCT TTGTCATATG CTGCTCTCCA 720
 ATTAAGAAC CAATCTGTGT CAATGTAGAG TTAAGTGCAG AGCAGTGAAG AAGAAAGTAT 780
 GAAAGAGAA AGGAAAGAAA TAAGACTCTA CGGAACACTA TTCAGTGGCT GGAAGACGAG 840
 CTAAACCGTT GCGTAACGG GAGGACAGTG CCTATTGATG AGCAGTTTGA CAAGAGAGAA 900
 GCTAATTTGG AAGCTTTCAC AGCGGATAA GATACTGCTA TTACCAGTGA TAAACGAGCT 960
 GCTGCAGTCG GAAATGGCTGG TAGTTTACC GATGCTGAAA GAAAGAAAGTG TGAAGAGAA 1020
 CTTGCTAAAT TGTATAACA GCTTGATGAC AAGTGAAG AGATTAAACA ACAAGGCCAA 1080
 TTGTTAGAGA AATTGAAGAC ACAATGCTG GATCAGGAAG AGCTTCTGGC ATCAAGCCAG 1140
 AGGGATCAAG ATATATGCA AGCTGAAGTG ATCGCTCC AAGCAQAAA TATGCTTCT 1200
 AAGAGAGAG TCAAGAGAGT TTTACAGGCC TTAGAGGAAC TGGCTGTTAA TTATGATCAG 1260
 AAGTCTCAGG AAGTTGAAGA CAACAACAG GAAATATGAT TGCTTAGTGA TGAATTGAA 1320
 CAAAATCTG CAATTTAGC AAGTATGAT GCTGAGCTTC AGAAGCTGA GGAATGACC 1380
 AACCAACAGA AGAACGAGC AGCTGAATG ATGGCATCAT TATTAAGAG CTTTGCAGAA 1440
 ATAGGAATTG CTGTGGGAA TAACGATGTG AAGCAACCA AGGAACCTGG TATGATAGAT 1500
 GAAGAGTTTA CTGTTGCAAG ACTCTACAT AGCAAAATGA ATCAGAAAT AAGAACCATG 1560
 GTGAACGCTG CAACACAGCT AGAAGCACG CAGACTGAGA GCAACAAA AATGGAAGAA 1620
 AATGAGAAAG AGTTAGCAGC ATGCCAGCTT CGGATCTCCC AACATGAAGC CAAAATCAAG 1680
 TCACTGACTG AGTACCTTCA GAATGTAGA CAAAAGAGA GGCAGCTGGA GGAATCTGTT 1740
 GATTCCCTTG GTAGGAGCT AGTCCAACCTC CGAGCACAG AGAAGTCCA TGAATCTGTT 1800
 AAGAGCACT TGAACAAGT TCAGACTGCA AATGAAGTCA AGCAAGCTGT TGAGCAGCAG 1860
 ATCCAGATC ACAGAGAAAC CCAACAAA CAATCAGTA GCTTCCGAGA TGAAGTTGAG 1920
 GCAAGGAAA AGCTAATCAC TGACCTCCA GACCAAAACC AGAAGATGGT GTTGGAGCAG 1980
 GAAAGGCTAA GGTGGAGCA TGAGAGGCTG AAGCTACAG ACCAAGAGAA GAGCAGGAAG 2040
 CTGCAAGAC TCACGGTTAT GCAAGACAGA CGAAGAACAG CAAGACAGA CTTGAAGGGT 2100
 TTGGAGGAGA CCGTGGCAA AAGAACTTCA ACTTTACACA ACCTGCCTAA GCTCTTTGTT 2160
 CAGGACTTGG CTACCAAGGT GAAAAGAGG CCGAGGTGCA CTCTGACGAC ACTGGCGGCA 2220
 GTGCTGCACA GAAGCAGAAA ATCTCTTCC TTGAAGACA CTTGAACAG CTCACCAAG 2280
 TGACCAAGCA GTTGGTAGCT GATAATGCAG ATCTTCGCTG TGAAGTTCTT AAGTTAGAGA 2340
 AACGGCTTAG AGCTACTGCA GAAAGAGTGA AAGCTTTGGA GTCAGCCCC 2389

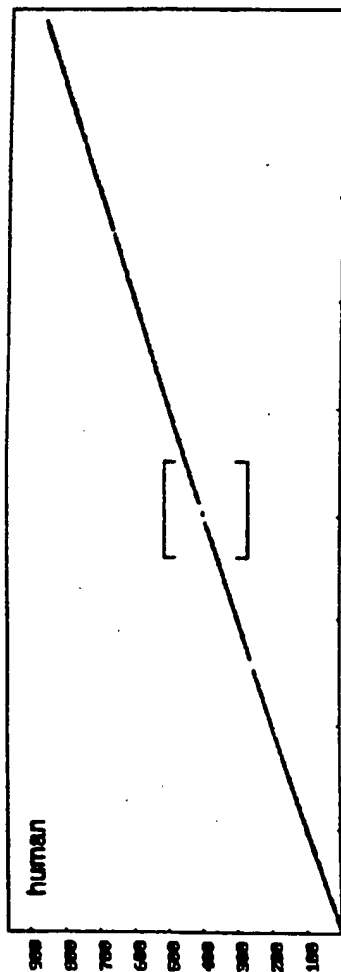
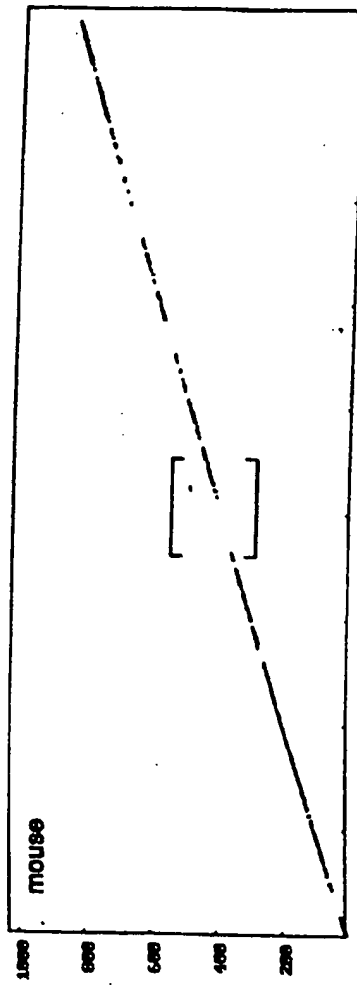
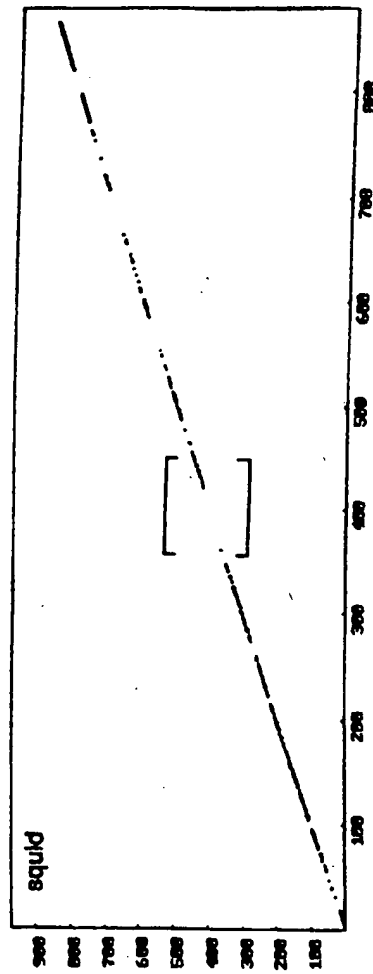
Figure 8A

Figure 8B



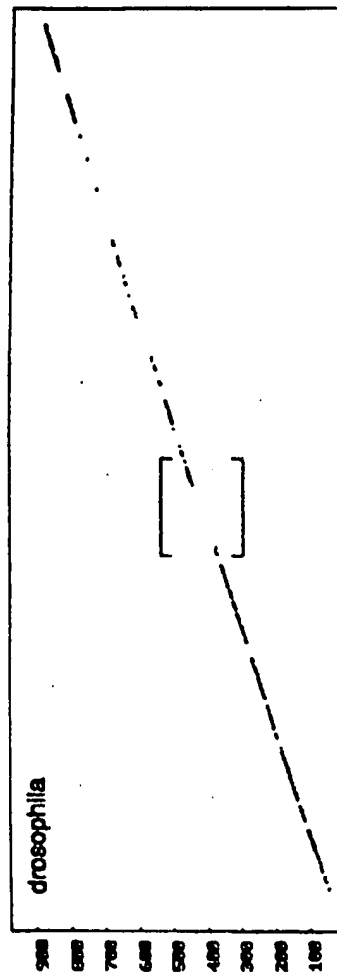
14 / 3 4

Figure 8C

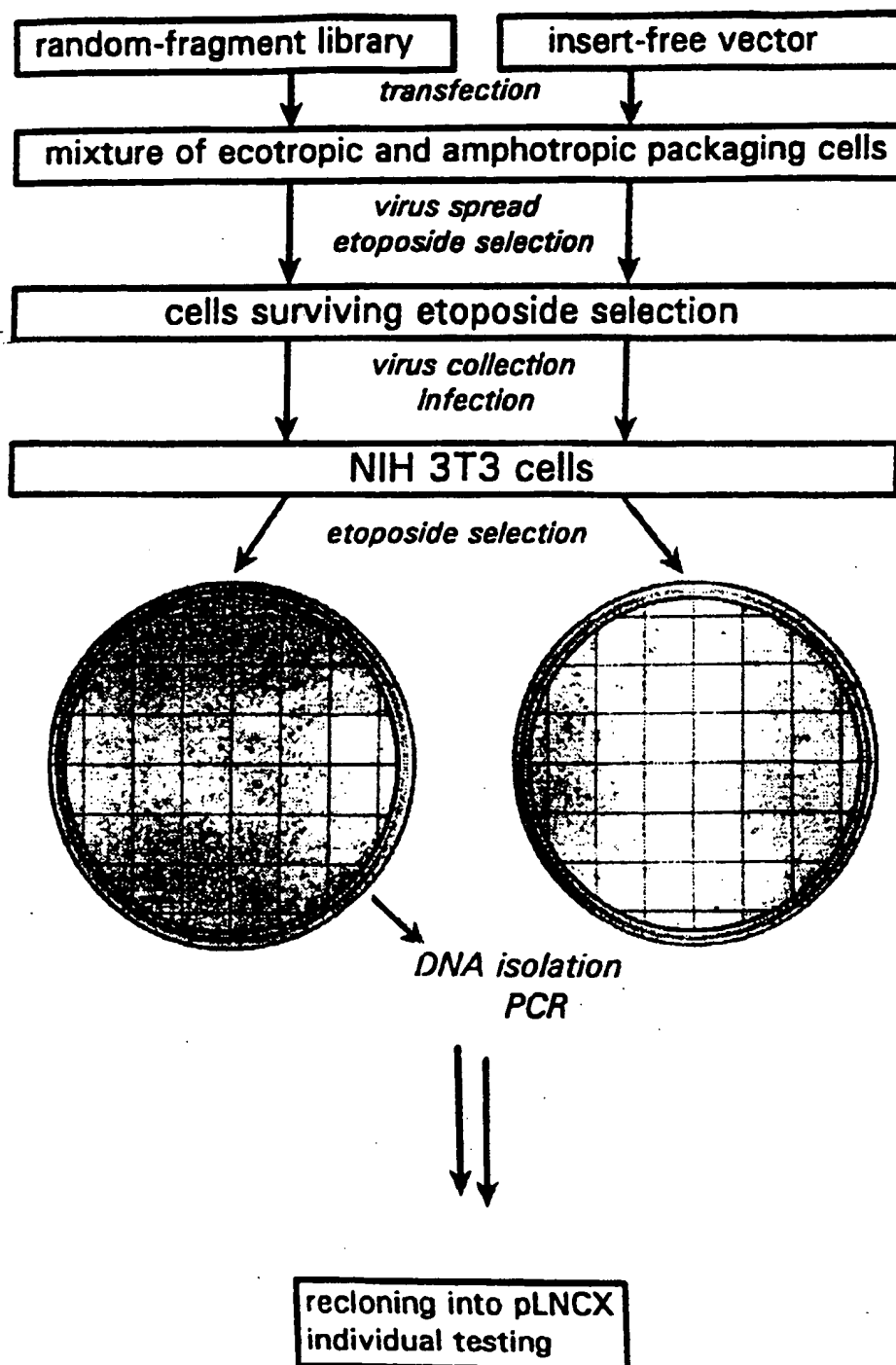


15 / 3 4

Figure 8D



16 / 3 4

**Figure 9A**

Hind III -ATG adaptor:

5'-CTCCCAAGCTTATGGATGGATG-3'
3'-AAAGAGGGTTCGAATACCTACCTAC-5'

Cla I-stop adaptor:

5'-TGAGTGAGTGAATCGATGATTAAA-3'
3'-ACTCACTCACTTAGCTACTAA-5'

Figure 9B

18 / 3 4

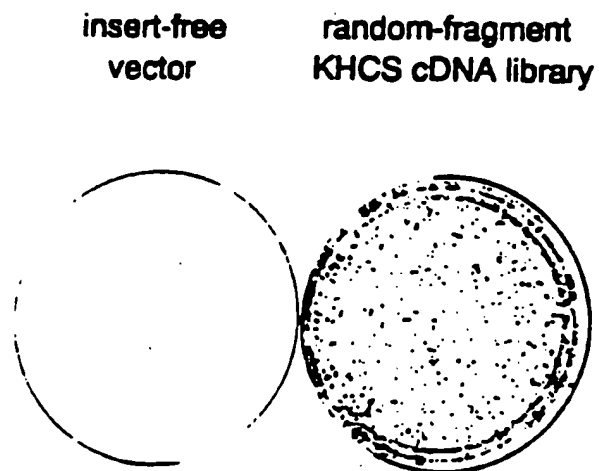
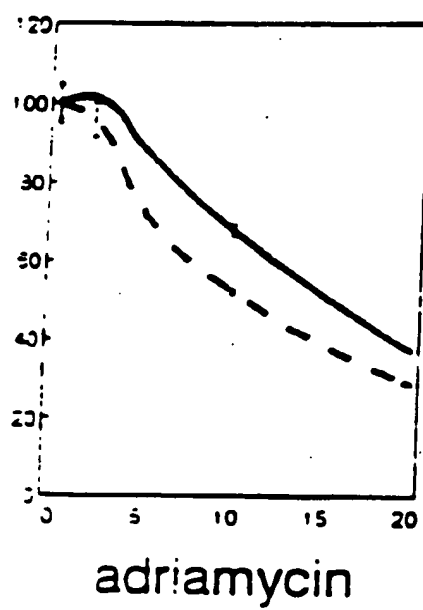


Figure 10

19 / 34

Figure 11A

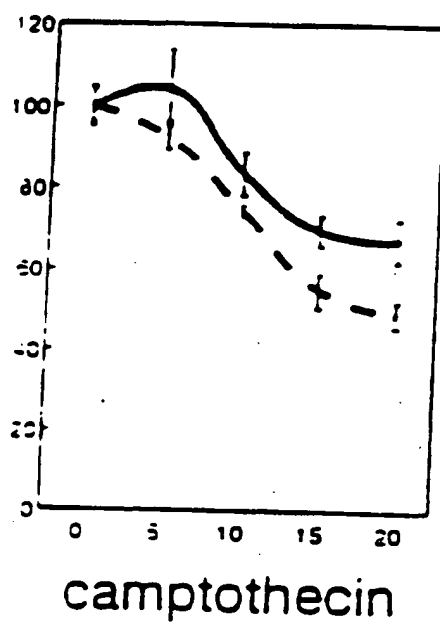
relative cell number (%)



20 / 34

Figure 11B

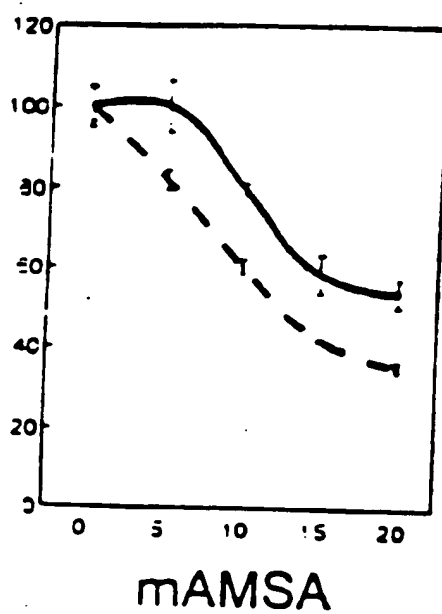
relative cell number (%)



21/34

Figure 11C

relative cell number (%)



22 / 34

Figure 11D

relative cell number (%)

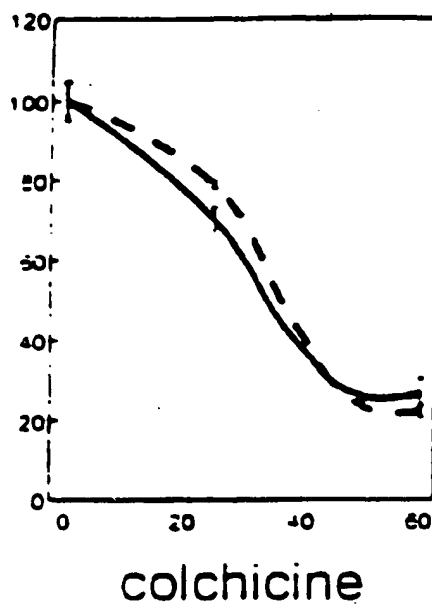
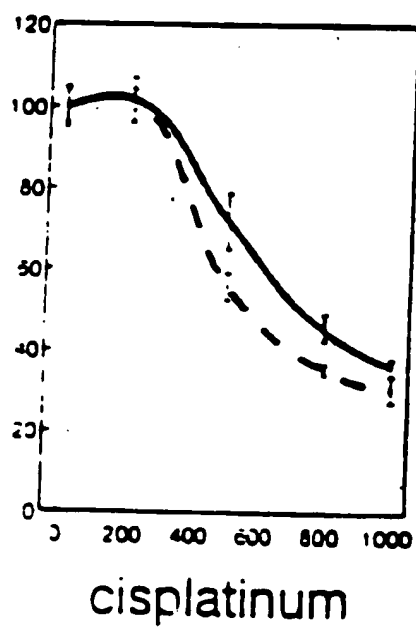


Figure 11E

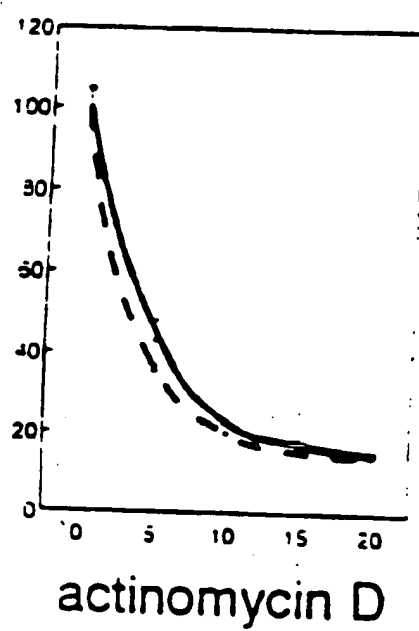
relative cell number (%)



24 / 34

Figure 11F

relative cell number (%)



25 / 34

Figure 11G

relative cell number (%)

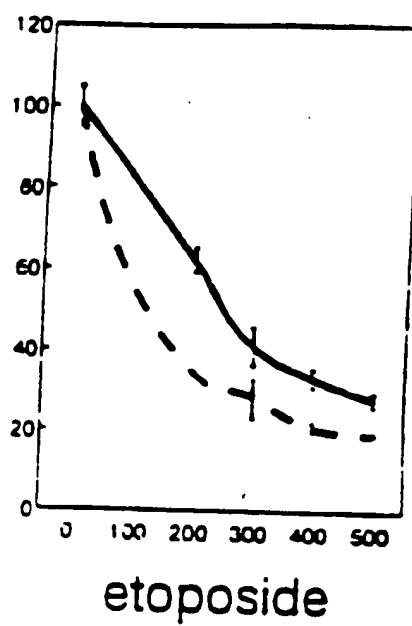
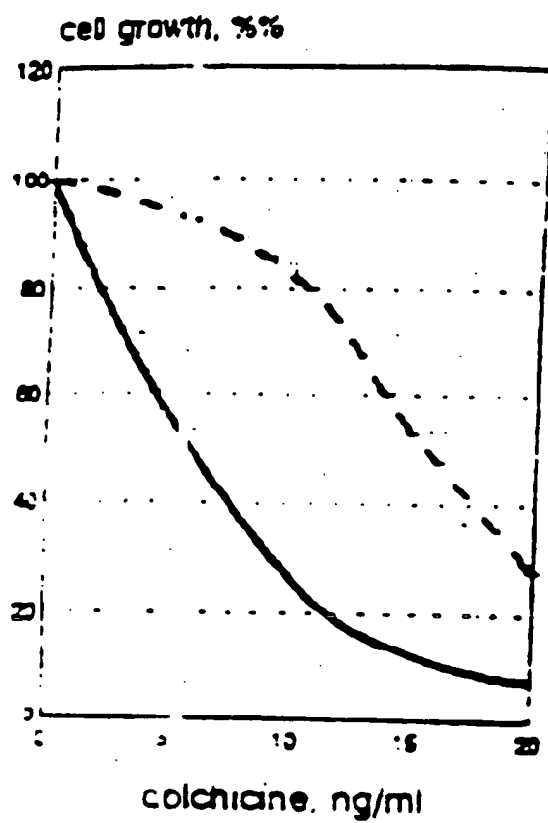
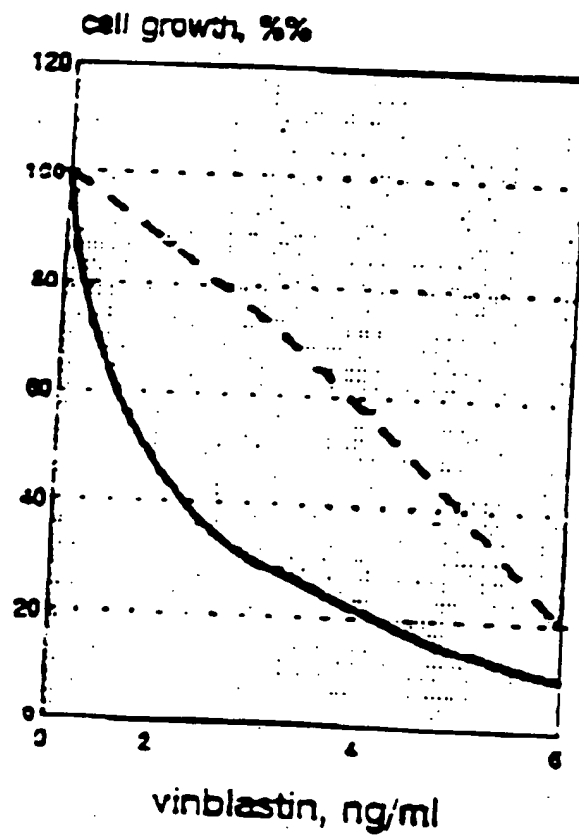


Figure 12A

———— anti-kinesin GSE

- - - - - control

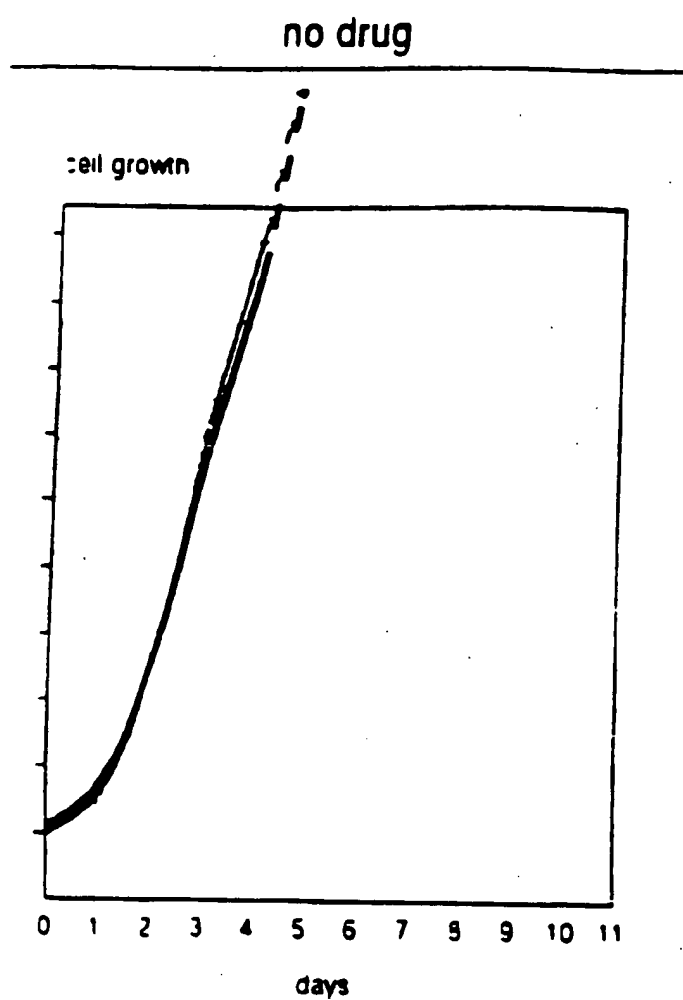
27 / 34

Figure 12B

— anti-kinesin GSE

- - - control

28 / 34

Figure 13A

29 / 34

Figure 13B

etoposide

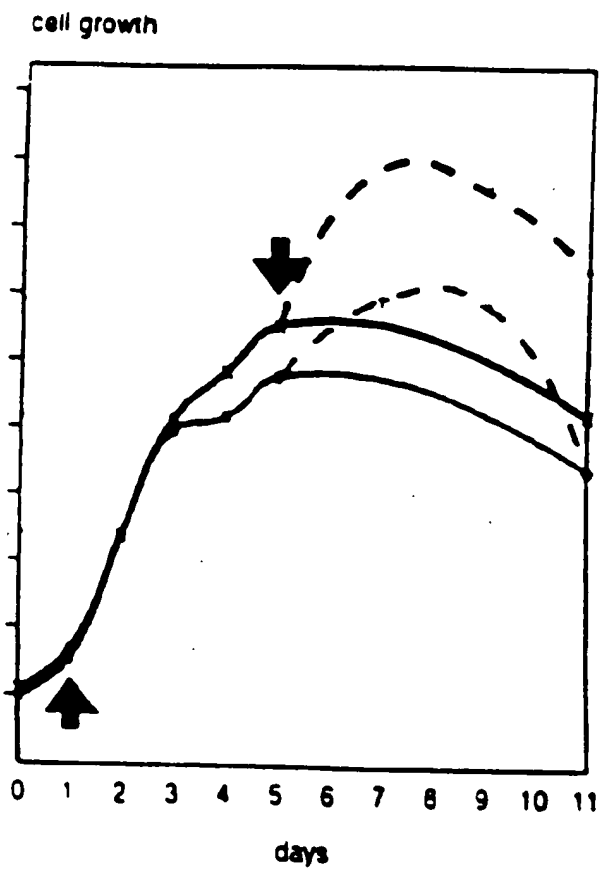
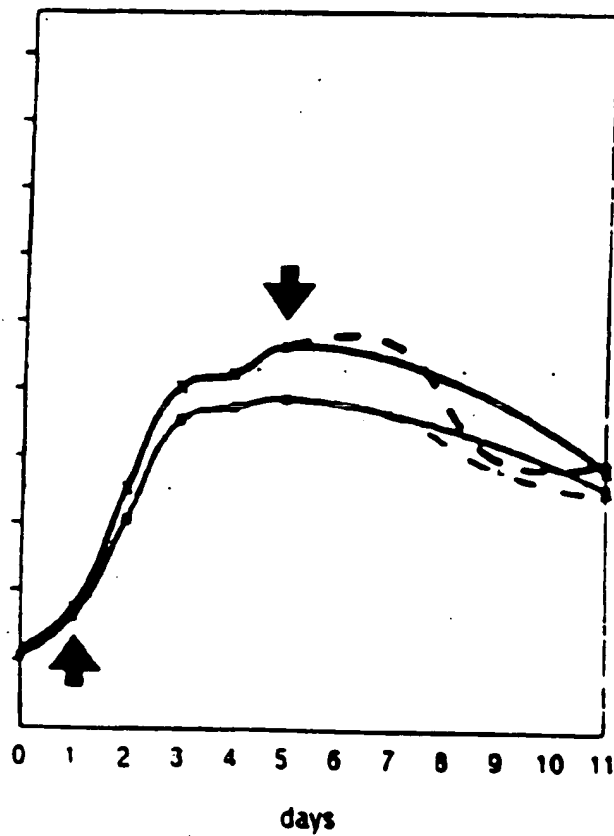


Figure 13C

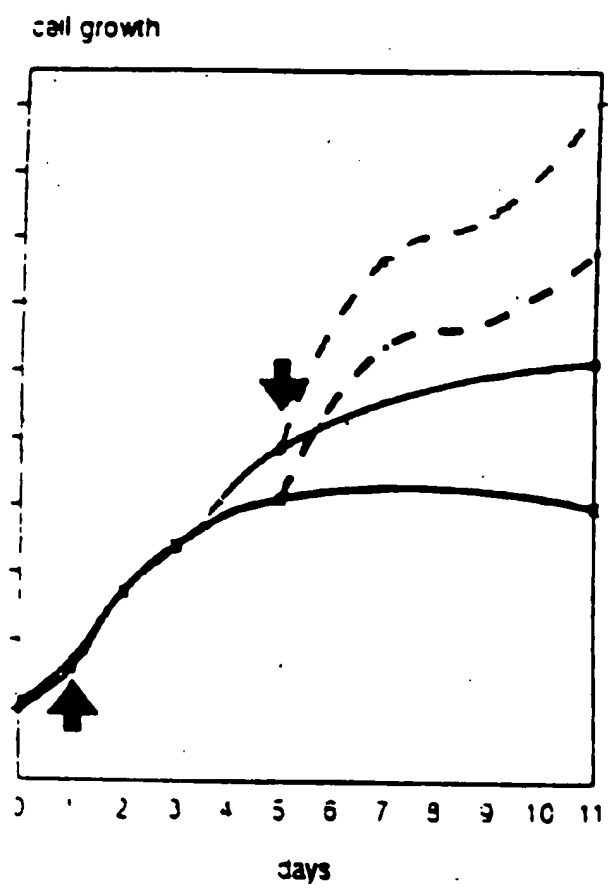
camptothecin

cell growth

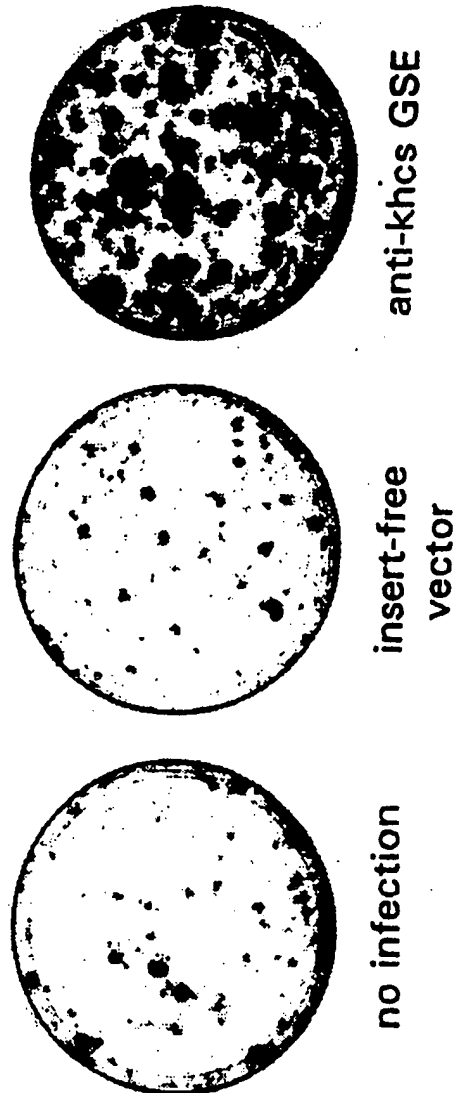


31 / 34

colchicine

**Figure 13D**

32 / 34

Figure 14

33 / 34

Figure 15



control

anti-KHCS GSE

3 4 / 3 4

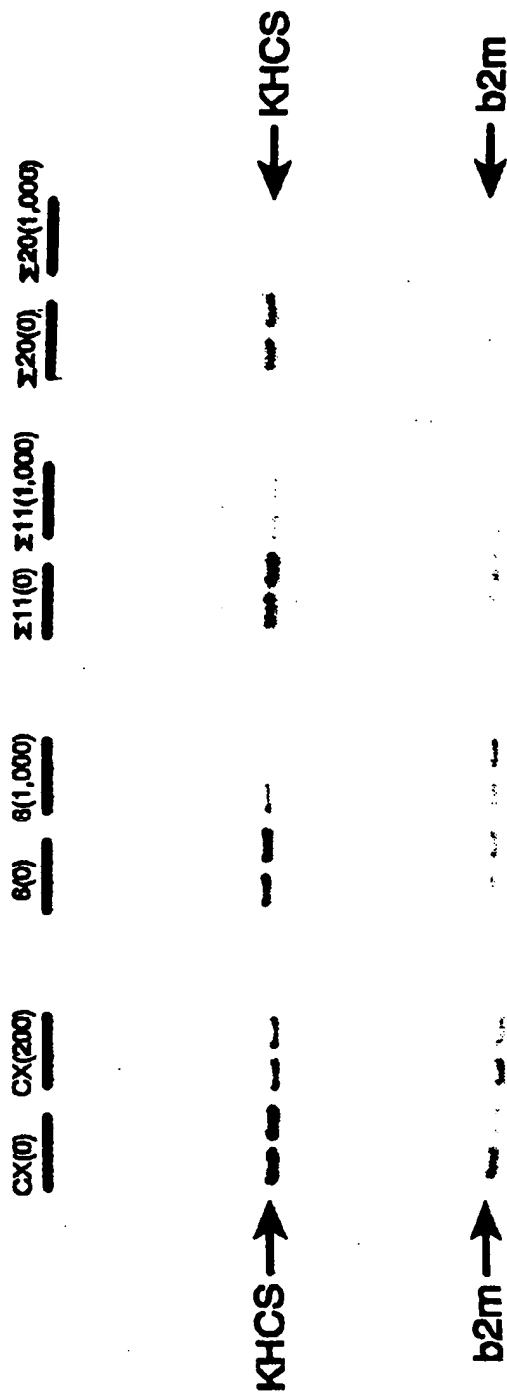


Figure 16

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 95/00092

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/47 A61K38/17 G01N33/68 C12Q1/68
C12N15/11 C12N5/10 //(C12N5/10, C12R1:91)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K G01N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO, A, 94 20618 (BOARD OF TRUSTEES OF THE UNIVERSITY OF ILLINOIS) 15 September 1994 cited in the application see claim 40; examples 4-6 ---	1-23
P, X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, no. 9, 26 April 1994, WASHINGTON US pages 3744 - 3748 GUDKOV A.V. ET AL. 'Cloning mammalian genes by expression selection of genetic suppressor elements: Association of kinesin with drug resistance and cell immortalization' see the whole document --- -/-	1-23

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

A document member of the same patent family

Date of the actual completion of the international search

13 April 1995

Date of mailing of the international search report

19.05.95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Espen, J

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		Relevant to claim No.
Category *	Citation of document, with indication, where appropriate, of the relevant passages	
P, X	PROCEEDINGS OF THE 'AMERICAN ASSOCIATION FOR CANCER RESEARCH, vol.35, March 1994 pages 652 - 653 RONINSON I. B. ET AL. 'Genetic suppressor elements: New tools for molecular oncology' see the whole document. ---	1-23
Y	NUCLEIC ACIDS RESEARCH, vol.20, no.4, 1992, ARLINGTON, VIRGINIA US pages 711 - 717 HOLZMAYER T. A. ET AL. 'Isolation of dominant negative mutants and inhibitory antisense RNA sequences by expression selection of random DNA fragments' see the whole document ---	1,15
Y	PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, vol.34, March 1993 page 538 GUDKOV A.V. ET AL. 'Genetic suppressor elements for cloning tumor suppressor genes' see abstract 3212 ---	1,15
X	THE JOURNAL OF CELL BIOLOGY, vol.117, no.6, June 1992 pages 1263 - 1275 NAVONE F. ET AL. 'Cloning and expression of a human kinesin heavy chain gene: Interaction of the COOH-terminal domain with cytoplasmic microtubules in transfected CV-1 cells' cited in the application see page 1267 - page 1270; figure 1 ---	2-5, 16-19
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol.90, April 1993, WASHINGTON US pages 3231 - 3235 GUDKOV A. V. ET AL. 'Isolation of genetic suppressor elements, inducing resistance to topoisomerase II-interactive cytotoxic drugs, from human topoisomerase II cDNA' cited in the application see the whole document ---	1,15
	--- -/--	

INTERNATIONAL SEARCH REPORT

Int'l. Application No.

PCT/US 95/00092

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol.88, March 1991, WASHINGTON US pages 1943 - 1947 PATANJALI S. R. ET AL. 'Construction of a uniform-abundance (normalized) cDNA' cited in the application see the whole document -----	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/00092

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 12-14 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/00092

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9420618	15-09-94	AU-B- 6444794	26-09-94